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Development and Application of Stable Isotope Dilution Assays for the *Fusarium* Mycotoxins Enniatins and Beauvericin

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Abstract

Enniatins (ENNs) and beauvericin (BEA) are termed “emerging *Fusarium* mycotoxins”, as data about occurrence and toxicology are scarce. In the present work, a stable isotope dilution assay (SIDA) for the determination of ENNs A, A1, B, B1 and BEA was developed, and applied to a variety of food samples.

The $^{15}N_3$-labeled ENNs and BEA were biosynthesized by feeding two *Fusarium* strains with $Na^{15}NO_3$ and subsequently isolated from the fungal culture by HPLC. Standard solutions of the $^{15}N_3$-labeled ENN A, ENN A1, and BEA were accurately quantitated by quantitative NMR. Using $^{15}N_3$-labeled ENNs and BEA as internal standards, a SIDA was developed, and the ENNs and BEA levels in various food samples were determined by LC-MS/MS. Analyses of cereals and cereal products revealed frequent contaminations of barley, wheat, rye, and oats with ENNs B and B1, whereas BEA was not quantifiable.

Subsequently, the newly developed SIDA was used to follow the fate of ENNs A, A1, B, B1 and BEA during beer making, which included malting and brewing. Three batches of barley grains were used as starting materials, one was naturally contaminated, two were artificially inoculated with *Fusarium* fungi. Samples were taken from each key step of the malting and brewing procedure. Significant increases of the toxins were found during germination of two batches of barley grains, resulting in green malt contamination up to a factor of 3.5 compared to grains before germination. After kilning, only 41–72% of the total amounts of the toxins in green malt remained in kilned malt. In the subsequent mashing stage, the toxins in kilned malt were predominantly removed with spent grains. In the final beer, only one batch still contained 74 and 14 μg/kg of ENN B and ENN B1, respectively. Therefore, the carryover of these ENNs from the initial barley to final beer was less than 0.2% with the main amounts remaining in the spent grains and the malt rootlets.

Furthermore, the SIDA was applied to study the effect of sourdough processing and baking on ENN B, ENN B1 and BEA concentrations. After milling of wheat and rye grains naturally contaminated with ENN B and ENN B1, approximately 70−82% of the two ENNs were found in the bran fraction, and the rest remained in flour. BEA was added to flour before sourdough fermentation, which lasted for 24 h at 30 or 40 ℃, and reduced part of the ENNs and BEA. Kneading, resting, and proofing of the

bread dough resulted in 13−19% reduction of the ENNs compared to flour, but in no significant change of BEA. The final baking at 200 ℃ for 25 min led to a further decrease of the ENNs and BEA, ranging from 9% to 28% compared to proofed dough. In case of rye sourdough bread, greater reductions of ENNs were found in crust than in crumb. Overall, the whole sourdough bread making process reduced 25–41% of ENN B, ENN B1 and BEA from the starting materials (flour and sourdough starter).

In addition, the contamination levels of ENNs A, A1, B, B1 and BEA in 60 Chinese medicinal herbs were examined by SIDA. Of all the analyzed samples, 25% were contaminated with at least one of the ENNs and BEA. BEA was the most frequently detected toxin with a 20% incidence in all samples. The percentages of ENN-positive samples were lower, each single ENN was detected in 6.7–11.7% of all samples. The total amounts of the five mycotoxins in single samples varied between 2.5 and 751 μg/kg, and the mean total amount in positive samples was 126 μg/kg.

Based on the contamination levels of ENNs and BEA in cereals and related products obtained from Germany in the present study, these mycotoxins are considered unlikely to be of human health concerns according to the threshold of toxicology concern (TTC) concept. The reduction of ENNs and BEA during sourdough bread making suggests that their toxic risks of sourdough bread would be generally lower than the original flour. Regarding the risks of ENNs and BEA from beer consumption, their low carryover from barley grains to beer reveals that they would pose little if any risk to beer drinkers.

Zusammenfassung

Enniatine (ENNs) und Beauvericin (BEA) sind Mykotoxine, die von der Pilzgattung *Fusarium* produziert und aufgrund unzureichender Daten zu Vorkommen und Toxikologie als „emerging toxins“ bezeichnet werden. In der vorliegenden Arbeit wurde eine Stabilisotopenverdünnungsanalyse (stable isotope dilution assay, SIDA) für die Bestimmung von ENNs A, A1, B, B1 und BEA entwickelt und auf verschiedene Lebensmittel angewendet.

$^{15}N_3$-markierte ENNs und BEA wurden durch Kultivierung von zwei *Fusarium* Stammen unter Zugabe von $Na^{15}NO_3$ biosynthetisiert und anschließend aus den Pilzkulturen mit HPLC isoliert. Standardlösungen der $^{15}N_3$-markierten ENN A, ENN A1, und BEA wurden mit Hilfe der quantitativen NMR sicher quantifiziert. Mit den $^{15}N_3$-markierten ENNs und BEA als internen Standards wurde eine SIDA entwickelt, und die Konzentrationen von ENNs und BEA in verschiedenen Lebensmittelproben wurden mittels LC-MS/MS bestimmt. Die Analyse von Getreiden und Getreideprodukten ergab eine häufige Kontamination von Gerste, Weizen, Roggen, und Hafer mit ENNs B und B1, wohingegen BEA nicht quantifizierbar war.

Anschließend wurde die neu entwickelte SIDA angewendet, um das Verhalten der ENNs A, A1, B, B1 und BEA während der Bierherstellung, d.h. dem Mälzen und Brauen, zu verfolgen. Drei Chargen einer Braugerste wurden als Ausgangsmaterialien eingesetzt, von denen eine natürlich kontaminiert und zwei künstlich mit *Fusarium* Pilzen beimpft waren. Von jedem Schlüsselschritt des Mälz- und Brauprozesses wurden Proben entnommen und analysiert. Signifikante Erhöhungen der Toxingehalte wurden während der Keimung in zwei Chargen der Gerste gefunden, was zu einer Kontamination von Grünmalz bis zum Faktor von 3.5 im Vergleich zumGehalt vor der Keimung führte. Nach dem Darren verblieben nur 41–72% von den gesamten Mengen der Toxine im Darrmalz. In den folgenden Maischprozessen wurden die Toxine in Darrmalz überwiegend mit dem Biertreber entfernt. Im fertigen Bier enthielt nur noch eine Charge Gerste jeweils 74 und 14 µg/kg von ENN B und ENN B1. Deshalb war die Übergangsrate der ENNs von den Ausgangsprodukten zum fertigen Bier weniger als 0.2%, währenddessen die größten Mengen in Biertreber und Malzmatrices zurückblieben.

Nachfolgend wurde die SIDA für Untersuchungen zum Einfluss der Sauerteigverarbeitung und des Backens auf die Konzentrationen von ENN B, ENN B1 und BEA verwendet. Nach dem Mahlen der Weizen- und Roggenkörner, die natürlich mit ENN B und ENN B1 kontaminiert waren, wurden circa

70−82% von den zwei ENNs in der Kleiefraktion nachgewiesen; der Rest blieb im Mehl zurück. BEA wurde vor der Sauerteig-Fermentation eingesetzt, die24 Stunden bei 30 oder 40 °C dauerte,und den Gehalt an ENNs und BEA reduzierte. Das Kneten, Stehenlassen und Fermentation des Sauerteigs führten zu einer 13−19%gen Reduktion von ENNs im Vergleich zum Mehl, ergab jedoch keine signifikante Veränderung von BEA. Das abschließende Backen bei 200 °C für 25 Minuten führte zu einer weiteren Reduktion von ENNs und BEA von 9% bis 28% gegenüber dem fermentierten Teig. Im Fall von Roggensauerteigbrot wurde eine stärkere Reduktion der ENNs in der Kruste als in der Krume nachgewiesen. Insgesamt reduzierte der ganze Sauerteigverarbeitungsprozess 25–41% der ENN B, ENN B1 und BEA aus den Ausgangsmaterialien (Mehl- und Sauerteigstarter).

Des Weiteren wurde die Kontamination von ENNs A, A1, B, B1 und BEA in 60 chinesischen Heilkräutern mit SIDA untersucht. von allen untersuchten Proben waren 25% mit mindestens einem der ENNs oder BEA kontaminiert. BEA kam am häufigsten vor mit einer Häufigkeit von 20% in allen Proben. Der Prozentgehalt der ENN-positiven Proben war niedriger, die einzelnen ENN wurden in 6.7–11.7% in den untersuchten Proben detektiert. Die gesamten Mengen von den fünf Mykotoxinen in den einzelnen Proben variierten zwischen 2.5 und 751 µg/kg, und die durchschnittliche Menge in positiven Proben lag bei 126 µg/kg.

Auf der Grundlage der ENN- und BEA-Gehalte in Getreide und Getreideprodukten, die in der vorliegenden Untersuchung ermittelt wurden, kann nach dem threshold of toxicology concern (TTC) Konzept ein Risiko für den Verbraucher als unwahrscheinlich beurteilt werden. Von der Reduktion von ENNs und BEA während der Herstellung von Sauerteigbrot kann abgeleitet werden, dass das toxikologische Risiko durch dieses Brot geringer ist als durch das Mehl, aus dem dieses Brot gebacken wurde. Der geringe Übergang von ENNs und BEA von Gerste ins Bier lässt ebenfalls auf ein vernachlässigbares Risiko für Biertrinker schließen.

Abbreviations

ACN	Acetonitrile
BEA	Beauvericin
bw	Body weight
CC_{50}	50% cytotoxic concentration
DON	Deoxynivalenol
DAD	Diode array detection
EC_{50}	50% effective concentration
ENN	Enniatin
ESI	Electrospray ionization
HPLC	High performance liquid chromatography
IC_{50}	50% inhibitory concentration
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LD_{50}	50% lethal dose
LOD	Limit of detection
LOQ	Limit of quantitation
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MeOH	Methanol
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
^{1}H-NMR	Proton nuclear magnetic resonance
qNMR	Quantitative nuclear magnetic resonance
rh	Relative humidity
Rt	Retention time
SIDA	Stable isotope dilution assay
SPE	Solid phase extraction
SNA	Spezieller Nährstoffarmer Agar
UV	Ultraviolet

1. Introduction

1.1 A brief overview of *Fusarium* mycotoxins

Mycotoxins are low molecular weight fungal metabolites, which are toxic to vertebrates and other animals at low concentrations (Rundberget et al., 2004; Desjardins et al., 1997). They are mainly produced by fungi belonging to three genera: *Aspergillus*, *Fusarium*, and *Penicillium* (Sweeney & Dobson, 1998). In the northern temperate regions, the most prevalent toxin-producing fungi are probably those of the *Fusarium* genus (EC-SCF, 2000). On a worldwide basis, trichothecenes, zearalenone, and fumonisins are the major *Fusarium* mycotoxins occurring in cereal grains, animal feeds, and forages (D'Mello et al., 1999).

1.1.1 Major *Fusarium* mycotoxins

1.1.1.1 Trichothecenes

Trichothecenes are a large group of cyclic sesquiterpenoids divided into four groups (types A–D), with type A and type B trichothecenes being the most common ones (Krska et al., 2007). The most prevalent trichothecenes are T-2 toxin and HT-2 toxin belonging to type A, and deoxynivalenol (DON), 3-acetyl-DON, 15-acetyl-DON, and nivalenol belonging to type B (Krska et al., 2001).

Global contamination of cereals and animal feed with trichothecenes has been reviewed by Placinta et al. (1999). DON and its acetylated derivatives as well as nivalenol were frequently detected, contamination of T-2 and HT-2 toxins was also reported, though less frequently.

The oral LD_{50} values for DON and 15-acetyl-DON were estimated to be 78 and 34 mg/kg bw, respectively, in B6C3F1 female mice (Forsell et al., 1987). The oral LD_{50} value for T-2 toxin was 10.5 mg/kg bw in mice (WHO, 1990).

1.1.1.2 Zearalenone

Zearalenone is a β-resorcyclic acid lactone, chemically defined as 3,4,5,6,9,10-hexahydro-14,16-dihydroxy-3-methyl-1*H*-2-benzoxacyclotetradecin-1,7(8*H*)-dione (Krska et al., 2007). In

animals and humans, zearalenone can be transformed into metabolites such as α-zearalenol, β-zearalenol, α-zearalanol, and β-zearalanol (JECFA, 2000).

Natural occurrence of zearalenone has been reported worldwide in cereals including corn, wheat, barley, oats, sorghum, and rice (Zinedine et al., 2007).

The toxicity of zeralenone and its metabolites is based on their estrogenic properties, as they are able to bind to estrogen receptors (Magan & Olsen, p.353, pp.356–357). The Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2000) has concluded that the safety of zearalenone could be evaluated based on the dose that had no hormonal effect in pigs, the most sensitive species, and established a provisional maximum tolerable daily intake (PMTDI) of 0.5 μg/kg bw for zearalenone.

1.1.1.3 Fumonisins

Fumonisins are a group of structurally related mycotoxins, mainly produced by *F. verticillioides* (formally *F. moniliforme*) and *F. proliferatum* (Kim et al., 2004). They are characterized by a 19- or 20-carbon aminopolyhydroxyalkyl chain that is diesterified with propane-1,2,3-tricarboxylic acid (Magan & Olsen, 2004, p.368).

The most naturally abundant fumonisins are fumonisins B1, B2, and B3 (Sydenham et al., 1997), they have been detected ubiquitously in corn and corn-based foods (Magan & Olsen, 2004, p.377).

Fumonisins cause leukoencephalomalacia, a fatal neurological disorder, in horses and donkeys, they are also toxic to liver, and part of their toxicological activity is demonstrated to be associated with disruption of sphingolipid biosynthesis (D'Mello et al., 1999).

1.1.2 Analytical methods of *Fusarium* mycotoxins

Among various methods for the analysis of *Fusarium* mycotoxins, thin-layer chromatography is cheap and simple, but less accurate compared to other methods such as HPLC and GC (Köppen et al., 2010). Fumonisins and type A trichothecenes cannot be analyzed by HPLC with UV detection due to lack of a UV-absorbing moiety, nor do they fluoresce. Therefore, they have to be derivatized before they can be analyzed by HPLC with fluorescence detection (Nelson, 1993; Asam & Rychlik, 2006). Zearalenone, however, can be analyzed by HPLC with direct fluorescence detection, which is the commonly used method, although GC methods are also available (Krska & Josephs, 2001). For type B

trichothecenes such as DON and nivalenol, HPLC-UV has been developed at a wavelength around 220 nm (Krska et al., 2001). Trichothecenes and fumonisins can also be determined by GC methods, but they also require time-consuming derivatization steps (Krska et al., 2001; Turner et al., 2009). In addition, enzyme-linked immunosorbent assay (ELISA) kits are available for DON, zearalenone, and fumonisin. However, ELISA is usually able to determine only one single mycotoxin at one time, and the kits are costly for bulk screening as they are for single use (Krska & Josephs, 2001; Turner et al., 2009).

In comparison, HPLC-MS/MS is possible to detect many mycotoxins in a single run without derivatization. Moreover, it is sensitive and accurate and, therefore, it has become the method of choice for mycotoxins analysis (Köppen et al., 2010). However, for quantitative determination based on HPLC-MS/MS methods, one issue must be addressed is the matrix effect. The latter may either decrease (in case of ion suppression) or increase (in case of ion enhancement) the intensity of analyte ions, thus affecting the accuracy and reproducibility of the assay (Matuszewski et al., 2003). Stable isotope dilution assay (SIDA) offers an ideal solution to matrix effect. Since the labeled internal standard and the analyte possess identical chemical and physical properties, both will be affected by matrix effect to the same extent.

During the sample preparation step of SIDA, the stable isotopically labeled (eg. ^{13}C, ^{15}N) internal standard is added to the sample, and equilibration between the isotopologues, i.e. the analyte and the internal standard, is reached. The term “dilution” is thus gained as the naturally abundant isotope in the analyte is diluted (Rychlik & Asam, 2008). If any loss of analyte happens in this step, it can be compensated for by identical loss of the internal standard. In subsequent LC-MS/MS measurement, the isotopologues can be distinguished due to their mass difference. Finally, the amount of analyte is calculated based on the peak area ratio between the analyte and internal standard, by referring to the response function between them, which have been determined beforehand. Usually, 1–1000 ng of labeled standard is added to each sample (Varga et al., 2012, Cramer et al., 2007, Asam & Rychlik, 2006) depending on the type of mycotoxin, the linear range of the response function, and contamination level.

In the recent decades, SIDAs for the analysis of different *Fusarium* mycotoxins have been developed either using commercially available total ^{13}C-labeled internal standards, or partially labeled (^{2}H, ^{13}C) internal standards, which were generated by chemical syntheses. Some examples of SIDAs for the analysis of *Fusarium* mycotoxins are given in **Table 1-1**.

Table 1-1. Determination of *Fusarium* mycotoxins by SIDAs with LC-MS/MS

Internal standard	LOD (µg/kg)	Matrix	Reference
[$^{13}C_2$]-monoacetoxyscirpenol	30	cereals, potato, feed	Asam & Rychlik, 2006
[$^{13}C_4$]-diacetoxyscirpenol	1		
[$^{13}C_2$]-HT-2 toxin	30		
[$^{13}C_4$]-T-2 toxin	3		
[$^{13}C_{15}$]-DON	2	cereals, feed	Asam & Rychlik, 2007
[$^{13}C_2$]-3-acetyl-DON	5		
[$^{13}C_2$]-15-acetyl-DON	4		
[$^{13}C_2$]-4-acetyl-nivalenol	1		
[$^{2}H_2$]-zearalenone	nd	cereals	Cramer et al., 2007
[$^{2}H_6$]-fumonisin B_1	0.4	corn, corn products	Lukacs et al., 1996
[$^{13}C_2$]-moniliformin	0.7	cereals	von Bargen et al., 2012

nd: not determined

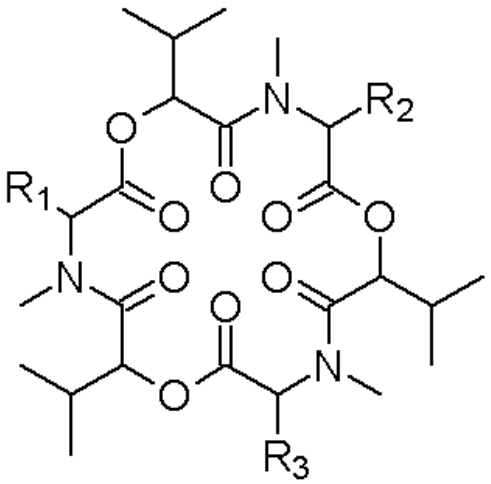
1.2 Emerging *Fusarium* mycotoxins ENNs and BEA

The emerging *Fusarium* mycotoxins ENNs and BEA are a group of structurally related cyclic hexadepsipeptides, they consist of three alternating D-α-hydroxyisovaleryl and three *N*-methylamino acid units (Blais et al., 1992; Xu, 1993; Hamill et al., 1969).

ENN was first isolated from the mycelium of *Fusarium orthoceras* var. *enniatinum*, and named by Gäumann et al. (1947). Although at least 27 ENNs have been reported (Fir áková et al., 2007) up to now, the most well-known ENNs as natural contaminants are ENNs A, A1, B, and B1 (Mahnine et al., 2011), therefore these 4 ENNs are in the focus of this study.

The structure of BEA was first reported by Hamill et al. (1969), who isolated the compound from the mycelium of the insect pathogen *Beauveria bassiana* (NRRL 3352), and named it BEA.

The structures of ENNs A, A1, B, B1, and BEA are presented in **Figure 1-1**. Individual ENNs are distinguished by the composition of different *N*-methylamino acid residues. ENNs A and B contain three isoleucine and valine residues, respectively, and ENNs A1 and B1 contain mixtures of these two residues (Blais et al., 1992; Xu, 1993), while BEA contains three phenylalanine residues (Hamill et al., 1969).



BEA: $R_1=R_2=R_3=$-$CH_2C_6H_5$

ENN A: $R_1=R_2=R_3=$-$CH(CH_3)CH_2CH_3$

ENN A1: $R_1=R_2=$-$CH(CH_3)CH_2CH_3$, $R_3=$-$CH(CH_3)_2$

ENN B: $R_1=R_2=R_3=$-$CH(CH_3)_2$

ENN B1: $R_1=R_2=$-$CH(CH_3)_2$, $R_3=$-$CH(CH_3)CH_2CH_3$

Figure 1-1. Chemical structures of ENNs A, A1, B, B1, and BEA

1.2.1 Production of ENNs and BEA by *Fusarium* fungi

ENNs are produced predominantly by various fungi of *Fusarium* species (Fir áková et al., 2007), BEA has also been reported being produced by a number of *Fusarium* fungi.

1.2.1.1 ENNs-producing *Fusarium* species

The most well-known producer of ENNs is *F. avenaceum*. Logrieco et al. (2002) reported that 13 strains of *F. avenaceum* isolated from Finnish wheat kernels were able to produce ENNs when grown on rice, with levels up to several thousand μg/g. Morrison et al. (2002) found that ENNs were produced by 24 *F. avenaceum* strains isolated from cereal kernels in Norway. Strongman et al. (1988), Blais et al. (1992), Uhlig et al. (2005), and Ivanova et al. (2006) also reported the production of ENNs by *F. avenaceum* grown either in cereal grains or (semi-)synthetic media.

Madry et al. (1983) investigated the influence of various nutritional factors on production of ENNs, and after optimization of the culture medium, ENNs yields up to 2.5 g/L were achieved by cultivating a strain of *F. oxysporum* in a chemically defined liquid medium. Moretti et al. (2002) found that ENN B alone, but not other ENNs, was produced by 11 strains of *F. oxysporum* grown on rice, with concentrations ranging from traces to 60 μg/g.

One *F. sambucinum* isolate produced 1.7 g/L of ENNs when grown in a liquid culture, which contained (w/v) 5% lactose and 0.8% tryptone (Audhya & Russell, 1974).

Also cultivated in chemically defined media, ENNs were produced by *F. acuminatum*, *F. arthrosporioides*, *F. lateritium*, *F. merismoides*, *F. scirpi*, *F. tricinctum*, as well as *F. avenaceum*, *F. oxysporum*, and *F. sambucinum* (Herrmann et al., 1996), and yields of ENNs ranged from 40 to 500 μg/mL. ENNs production by *F. tricinctum* was also reported by Burmeister & Plattner (1987) and Wätjen et al. (2009).

Moretti et al. (2007) reported production of ENNs by *F. proliferatum*, *F. acutatum*, and *F. denticulatum* grown on rice. Strains of *F. poae*, *F. langsethiae*, and *F. sporotrichioides* were found to produce ENNs when cultivated on potato sucrose agar, yeast extract sucrose agar, maize kernels, or rice (Thrane et al., 2004).

1.2.1.2 BEA-producing *Fusarium* species

Gupta et al. (1991) reported the first isolation of BEA from the genus *Fusarium*, i.e. *F. semitectum* and *F. moniliforme* var. *subglutinans*.

The species *F. subglutinans* was frequently found to produce BEA. Logrieco et al. (1993a) reported the production of BEA by 8 maize isolates of *F. subglutinans* from Peru, the yields varied from 50–250 mg per kg corn kernels, which were used as culture medium. Similar amounts (up to 200 mg/kg) of BEA were produced by *F. subglutinans* isolates from Poland when cultured on corn kernels (Logrieco et al., 1993b). When inoculated on maize kernels, 4 Austrian isolates of *F. subglutinans* produced 176–687 mg/kg of BEA (Krska et al., 1997). Production of BEA by isolates of *F. subglutinans* from South Africa (Shephard et al., 1999), Slovakia (Srobarova et al., 2002), Canada (Moretti et al., 1995) have been reported, too.

F. proliferatum is also a well-known producer of BEA. Plattner & Nelson (1994) found that 300–1100 mg/kg BEA were produced by 3 strains of *F. proliferatum* grown on cracked corn. Four South African isolates of *F. proliferatum* produced similar amounts of BEA (Shephard et al., 1999) on corn kernels. In a sample of *F. proliferatum*-inoculated cracked corn, 330 μg/g of BEA was detected (Thakur et al., 1997). An Austrian isolate of *F. proliferatum* produced 266 mg/kg BEA on maize kernels (Krska et al., 1997).

F. oxysporum is another species often reported to produce BEA. Moretti et al. (2002) studied 44 strains of *F. oxysporum*, and 36 strains were able to produce BEA from 1 to 310 μg/g when grown on rice kernels. Three strains of *F. oxysporum* from Poland produced up to 3200 μg/g BEA on maize kernels (Logrieco et al., 1998).

Logrieco et al. (1998) also reported on the production of BEA by 12 strains of *F. sambucinum*, as well as other species such as *F. anthophilum*, *F. dlamini*, *F. longipes*, and *F. poae*.

Other *Fusarium* species including *F. moniliforme* (Plattner & Nelson, 1994), *F. avenaceum* (Logrieco et al., 2002; Morrison et al., 2002), *F. circinatum*, *F. concentricum*, *F.guttiforme* (Fotso et al., 2002), *F. fujikuroi*, *F. bulbicola*, *F. denticulatum*, *F. lactis*, *F. phyllophilum*, *F. pseudocircinatum*, and *F. succisae* (Moretti et al., 2007) were also able to produce BEA.

1.2.2 Occurrence of ENNs and BEA in food

The natural occurrence of ENNs ad BEA has been reported in cereals such as maize, barley, wheat, rice, and oats, as well as in cereal-based food.

In Poland, 14 samples of preharvest corn collected in 1990–1991 were contaminated with 5–60 mg/kg of BEA (Logrieco et al., 1993b). Up to 520 mg/kg of BEA was found in moldy maize ears collected at preharvest time in 1994 in Italy (Ritieni et al., 1997). In Denmark (Sørensen et al., 2008), 100% of the 43 maize samples harvested in 2006 was contaminated with at least one of the ENNs (up to 2598 μg/kg) and 98% contained BEA (up to 988 μg/kg). Maize samples naturally contaminated with BEA were also found in South Africa (Sewram et al., 1999) and Croatia (Jurjevic et al., 2002).

Logrieco et al. (2002) examined 13 wheat samples (each containing 100 kernels) affected by head blight in Finland, all were contaminated with BEA (0.64–3.5 mg/kg), and at least one of the ENNs was detected in 12 samples (level of single ENN up to 6.9 mg/kg).

Jestoi et al. (2004) determined the contamination levels of 30 cereal-based products purchased from Finnish and Italian markets, 97% and 57% were positive with ENNs and BEA, respectively. Overall, the contamination levels were low, total ENNs levels were no more than 271 μg/kg, and BEA was below 10 μg/kg.

A survey conducted by Uhlig et al. (2006) on Norwegian grains revealed high prevalence of ENNs and BEA. Of the 228 grain samples (oats, barley, and wheat), 100% and 32% were contaminated with ENNs and BEA, respectively. Moreover, concentrations of ENN B above 1000 μg/kg were commonly detected in barley and wheat.

Jestoi et al. (2009) investigated a number of egg samples for residues of ENNs and BEA. Of the 62 samples in 2004 and 50 samples in 2005 obtained from the Finnish residue control programme, 79% (in 2004) and 56% (in 2005) were positive with ENNs and/or BEA, but the contamination levels were all below 1.12 μg/ kg. Moreover, of the 367 egg samples purchased from Finnish markets in 2005, 366 were contaminated with ENNs and/or BEA, the highest level of single toxin was 7.5 μg/ kg.

Sixty-four cereal samples from the community of Valencia in Spain were analyzed by Meca et al. (2010c), 73.4 and 32.8% were contaminated with ENNs and BEA, respectively. Mean levels of total ENNs in corn, wheat, and barley samples were all above 148 mg/kg. ENN A1 levels above 810 mg/kg were detected in 2 samples (rice and corn). The BEA levels were relatively lower, with mean levels of 2.3–5.7 mg/kg in corn, wheat, and barley samples, and a maximum level of 11.78 mg/kg in rice. The same group (Mahnine et al., 2011) also examined 68 cereal products from Morocco, using the same analytical method to that of Meca et al. (2010c). Twenty out of 48 breakfast cereals samples were contaminated with ENNs, a maximum total ENNs level of 1264 mg/kg was found in a wheat product; and 2 out of 20 infant cereals samples were ENNs-positive. The incidence of BEA was lower than that of ENNs, 4 samples were BEA-positive out of the total 68 samples, the maximum level was detected in a rice-based infant cereal sample (10.6 mg/kg). However, considering the high LOQs (400–600 μg/kg) of the method used in these two reports, the real frequency of positive samples might be higher if a more sensitive method were used.

1.2.3 Biological activities of ENNs and BEA

ENNs and BEA possess a wide range of biological activities such as antimicrobial, insecticidal, and cytotoxic activities, and these activities are suggested to be related to their ionophoric properties (Shemyakin et al. 1969; Krska et al., 1996; Jestoi et al., 2009).

Ionophoric activity:

ENNs and BEA have been reported to act as ionophores, which transport metal ions (eg. K^+, Na^+, Ca^{2+}) through lipid membranes (Shenyakin et al., 1969; Prince et al., 1974; Roeske et al., 1974; Benz, 1978). ENNs and BEA are able to bind to alkali metal and alkaline earth metal ions, forming 1:1, 2:1 and 3:2 ENN/BEA:cation complexes. In the 1:1 complex, the ion is accommodated in the molecular cavity; in the 2:1 and 3:2 complexes the cation is sandwiched in between two molecules (Ovchinnikov et al., 1974).

Due to their hydrophobicity, ENNs easily incorporate into the cell membrane in which they form cation-selective pores, and act as passive channels (Kamyar et al., 2004). In an investigation of the channel-forming activity of BEA in ventricular myocytes and synthetic membranes, Kouri et al. (2003) found that BEA forms cation-selective channels in lipid membranes.

Phtotoxicity:

Growth reduction of germinating wheat seeds was observed when ENN B (10–80 μg/mL) was added to the solution in which the seeds were germinated (Burmeister & Plattner, 1987). The reduction was found to be concentration dependent, and root elongation was retarded to a greater extent than leaf elongation.

Herrmann et al. (1996) found that a mixture of ENNs (ENNs A, A1, B, and B1) caused necrosis of potato tissue, when applied at levels of 10 μg or higher per slice of potato.

The effects of ENN B on seed germination of the parasitic weed *Striga hermonthica* was evaluated by Zonno & Vurro (1999). After 36 h of exposure to ENN B at 0.1 μM concentration, 52.8% of the seeds were inhibited from germinating.

Antimicrobial activity:

Tomoda et al. (1992b) reported that ENNs A, A1, B1, and BEA showed weak activity against Gram-positive bacteria *Staphylococcus aureus*, *Bacillus subtilis*, and *Bacillus cereus*, with MIC (minimum inhibitory concentration) values of 12.5–50 μg/mL. The activity of ENNs against the bacterium *Micrococcus luteus* was more potent, with MIC of 1.56–6.25 μg/mL.

ENNs A1, B, and B1 exhibited potent antifungal activity against the plant pathogen *Eutypa armeniacae*, showing inhibition zones of 8–14 mm when 8 to 170 μg of the ENNs were applied to each disk where the fungus was inoculated (Tsantrizos & Xu, 1993).

The antifungal activity of ENNs to the phytopathogen *Botrytis cinerea* was determined by Pohanka et al. (2004), and a minimum inhibitory concentration of 75 μg/mL to spore germination was found for ENN B1.

Jayasinghe et al. (2006) observed moderate activity of ENNs A, A1, and B1 against the fungi *Candida albicans* and *Cryptococcus neoformans*, as well as against the bacterium *Mycobacterium intracellulare*, with IC_{50} values between 2.0 and 15.0 μg/mL.

The antifungal effect of ENN B was tested with *Beauveria bassiana* and *Trichoderma harzianum* inoculated on disks (Meca et al., 2010b). The growth of the moulds was inhibited by ENN B with the smallest amounts of 5 and 1 μg per disk, respectively.

Insecticidal activity:

ENN A, BEA, and a mixture of ENN A1, B, and B1 were found to show insecticidal activity towards adults of the blowfly *Calliphora erythrocephala*, and larvae of the mosquito *Aedes aegypti* (Grove & Pople, 1980), LD_{50} values were not given.

The extract (major component: ENNs A and A1) from hyphae of *Fusarium avenaceum* exhibited toxicity to spruce budworm larvae (Strongman et al., 1988).

Gupta et al. (1991) reported that BEA was toxic to Colorado potato beetle (*Leptinotarsa decemlineata*), with an LC_{50} of 633 ppm.

Pleiss et al. (1996) reported the anthelmintic activity of ENN A, which was fully active against adult *Nippostrongylus brasiliensis* worms and *Trichinella spiralis* larvae at a concentration of 5 μg/mL. In

contrast to this, ENNs A1, B, and B1 were less effective, and BEA possessed even less anthelmintic potency.

Cytotoxicity:

Cytotoxicity is the most extensively studied activity of ENNs and BEA. These mycotoxins have been reported to exert cytotoxic effects to various cell lines.

Inhibition of L-type Ca^{2+} current by BEA in the NG108-15 neuronal cell line (a mouse neuroblastoma and rat glioma hybrid cell line) was demonstrated by Wu et al. (2002). The inhibition was reversible and concentration dependent, with an IC_{50} of 4 µM.

Calò et al. (2004) reported the cytotoxic effects of BEA to two human cell lines of myeloid origin: the monocytic lymphoma cells U-937 and the promyelocytic leukemia cells HL-60. After 24 h exposure to BEA, the CC_{50} values assessed by the trypan blue exclusion method were 30 and 15 µM for U-937 and HL-60 cells, respectively.

The cytotoxicity of BEA and a mixture of ENNs (ENNs A, A1, B, and B1) to lepidopteran (*Spodoptera frugiperda*) cell line (SF-9) were determined by Fornelli et al. (2004). The cell viability was assessed by both the trypan blue exclusion and the MTT assays, with an incubation time of 48 h. By trypan blue exclusion assay, BEA and ENNs exhibited CC_{50} values of 3.0 and 6.7 µM, respectively, whereas the major *Fusarium* mycotoxins zearalenone and DON showed CC_{50} values of 18.3 and 45.0 µM, respectively. By MTT assay, the IC_{50} values for BEA and ENNs were 2.5 and 6.6 µM, respectively, which were also lower than those of zearalenone (17.5 µM) and DON (47.6 µM).

BEA was also found to induce cell death in human leukemia cells in a dose- and time-dependent manner (Jow et al., 2004). The IC_{50} value in the 24 h treatment was 2.5 µM. The authors speculated the process was likely to undergo through an apoptotic pathway.

Ivanova et al. (2006) investigated the cytotoxicity of ENNs A, A1, B, B1 and BEA in two cell lines of human origin, i.e. the hepatocellular carcinoma-line Hep G2 and the fibroblast-like foetal lung cell line MRC-5. The metabolic activity of the cells was tested by Alamar Blue™ (a redox-indicator) assay. Cell proliferation was measured by BrdU-assay based on quantification of DNA synthesis. The IC_{50} values for ENNs A, A1, B1, and BEA were all in the lower micromolar-range, with a lowest value of

0.8 µM. The cytotoxicity of ENNs was comparable to that of DON in the BrdU assay, while in the Alamar Blue™ assay, DON was significantly more toxic.

Cytotoxicity of ENNs (a mixture of ENNs A, A1, B, and B1 at µM concentrations) against a number of human cell lines was conducted with MTT assay (Dornetshuber et al., 2007). The ENNs were found to exhibit profound p53-dependent cytostatic and p53-independent cytotoxic effects against several human tumor cell lines including melanoma, carcinoma, and leukaemia cell lines.

Wätjen et al. (2009) determined the toxic effects of ENNs A1, B and B1 on a few cancer cell lines by MTT assay. The ENNs showed moderate activity in HepG2 human hepatoma cells and C6 rat glioma cells (EC_{50} values = 10–25 µM), and high toxicity in H4IIE rat hepatoma cells (EC_{50} values = 1–2.5 µM). Apoptotic cell death was observed in H4IIE cells with all three ENNs at concentrations of 1 and 2.5 µM.

ENN B was reported to exert pronounced cytotoxic effects in V79 cells (lung fibroblasts from Chinese hamster). Though less potent than DON (IC_{50} = 0.8 µM), ENN B exhibited an IC_{50} of 4 µM after 48 h exposure determined by neutral red uptake assays (Behm et al., 2009). As ENN B is stable in the gastrointestinal tract, the authors assumed it may thus exert prolonged effects upon ingestion.

The cytotoxicity of ENNs to Caco-2 cells (human epithelial colorectal adenocarcinoma cells) was determined by MTT assay (Meca et al., 2010a). ENNs A1 and B1 were found to be toxic with IC_{50} values of 12.3 and 19.5 µM, while ENNs A and B were not effective at the tested concentrations (0.6–30 µM).

Cytotoxicity of BEA to mammalian kidney epithelial (Vero) cells was evaluated by neutral red and MTT assays (Ruiz et al., 2011b). After incubation with BEA, IC_{50} values of 6.77–11.08 and 6.25–10.02 µM were found by neutral red and MTT assays, respectively. These values were comparable to those of DON, which showed IC_{50} values of 3.30–10.00 and 5.05–8.02 µM by neutral red and MTT assays, respectively. The same group (Ruiz et al., 2011a) also investigated the cytotoxic effects of BEA to immortalized hamster ovarian cells (CHO-K1). By neutral red assay, after 24, 48, and 72 h exposure, the IC_{50} values of BEA were 17.2, 6.2, and 3.8 µM, respectively, whereas the IC_{50} values of DON were 2.3, 1.9, and 1.8 µM, respectively. Similar values were obtained by MTT assay.

Tonshin et al. (2010) investigated the mechanisms of cyototoxicity caused by ENNs using isolated rat liver mitochondria. They found that the toxic effects were strongly connected with the potassium (K^+)

ionophoric activity of the ENNs, which were highly selective for potassium ions. Therefore, the mitochondrion and the homeostasis of potassium ions were suggested to be the cytotoxicity targets of ENNs.

Electromechanical and electrophysiological properties:

Lemmens-Gruber et al. (2000) studied the electromechanical and electrophysiological effects of BEA in isolated smooth and heart muscle preparations of guinea pig. Decreased force of contraction in precontracted terminal ilea was observed with an IC_{50} of 0.86 µM, and in electrically stimulated papillary muscles an IC_{50} of 18 µM was found.

Later, Kamyar et al. (2004) reported the electromechanical and electrophysiological effects of ENNs in isolated preparations of guinea pig. A mixture of ENNs A, A1, B, and B1 was found to decrease the force of contraction in electrically driven papillary muscles and precontracted terminal ilea, with an IC_{50} of 2.9 µM.

Other activities:

Tomoda et al. (1992a) studied the inhibition of acyl-CoA:acyltransferase (ACAT) by BEA and ENNs. In an enzyme assay using rat liver microsomes, BEA showed an IC_{50} of 3.0 µM, while ENNs exhibited much higher IC_{50} values of 22 to 110 µM. In a cell assay using J774 macrophages, BEA showed the highest CD_{50} (the drug concentration causing 50% cell damage)/IC_{50} value of 65, indicating BEA also was the most specific ACAT inhibitor of microbial origin.

McKee et al. (1997) evaluated the *in vivo* toxicity of ENNs A1, B, and B1 towards mice. Most deaths occurred between days 2 and 3 with the 40 mg/kg bw dose, and with the 20 and 10 mg/kg bw dose, death occurred between days 4 and 5. The authors concluded there was no significant *in vivo* activity of the ENNs.

1.2.4 Analytical methods of ENNs and BEA

Different analytical methods for the determination of ENNs and BEA have been developed. Among them HPLC with DAD or MS (including tandem MS) detection are the most often used. As the maximum UV absorption of ENNs and BEA occurs at low wavelengths, DAD detection is usually carried out between 192–209 nm (Krska et al., 1996, Josephs et al., 1999), which makes it vulnerable to interference of coeluting compounds. In contrast to this, HPLC coupled with MS or MS/MS detection is more specific and sensitive, and different MS interfaces such as ESI, APCI, and thermospray have been used (Sewram et al., 1999; Uhlig & Ivanova, 2004; Thakur & Smith, 1997). Several representative methods are shown in **Table 1-2** for comparison.

Table 1-2. Several representative analytical methods for determination of ENNs and BEA

	Detection	LOD (μg/kg)	Recovery	Matrix
Krska et al., 1996	HPLC-DAD, 192 nm	50 (BEA)	83%	corn
Josephs et al., 1999	HPLC-DAD, 209 nm	46 (BEA)	96%	corn, cornmeal
Sewram et al., 1999	LC-MS	0.5 (BEA)	94%	maize
Monti et al., 2000	HPLC-DAD, 205 nm	1.2–20	56–76%	water
Uhlig & Ivanova, 2004	LC-MS/MS	3.0–4.0	73–131%	oat, wheat, baley
Sørensen et al., 2008	LC-MS/MS	7–13	68–173%	maize, maize silage
Jestoi et al., 2009	LC-MS/MS	0.015–0.56	48–79%	eggs
Mahnine et al., 2011	HPLC-DAD, 205 nm	140–215	85–89%	cereals

1.3 Effect of food processing on mycotoxins

Many cereals and other crops are susceptible to attack by fungi either in the field or during storage, these fungi may produce mycotoxins which cause health problems (Magan & Olsen, 2004, p.1). On the other hand, food processing of cereals such as milling, brewing, baking, frying, and roasting may have effects on mycotoxins (Bullerman & Bianchini, 2007), possibly leading to less contaminated food than the raw materials. Therefore, it is important to know the changes of mycotoxins during specific food processing in order to assess their potential risk to humans.

1.3.1 Malting and brewing

Beer is a popular and widely consumed drink in the world. In some countries such as the Czech Republic, Germany and Austria, the annual per capita consumption exceeds 100 L (Beer statistics, 2010). Unfortunately, the major raw material for beer production, barley, is frequently infected with mycotoxin-producing fungi (Medina et al., 2006). Some residues of the mycotoxins accumulated in barley grains may survive the beer production chain and contaminate the final product.

The beer production process mainly includes malting and brewing. Some earlier studies have focused on the fate of aflatoxin, ochratoxin A, zearalenone, fumonisins, as well as DON and 15-acetyl-DON during the beer making process (Chu et al., 1975; Krogh et al., 1974; Scott et al., 1995; Schwarz et al., 1995). Aflatoxin B1 and ochratoxin A were found to be partially removed during the brewing process (eg. removal with malt mash), only 14–18% and 27–28%, respectively, of the two mycotoxins were found in final beer (Chu et al., 1975). Scott et al. (1995) reported that 2–13% of ochratoxin A, 2–28% of fumonisin B1, and 9–17% of fumonisin B2 were reduced during the fermentation step. Levels of zearalenone, DON, and 15-acetyl-DON were found to increase during malting, due to mold growth (Schwarz et al., 1995), and the majority of DON was still present in final beer.

In recent years, more detailed studies regarding the fate of DON and its derivatives have been carried out due to their predominance in beer (Lancova et al., 2008b; Kostelanska et al., 2011b). The authors studied the influence of the key steps such as steeping, germination, kilning, mashing, and fermentation on the behavior of mycotoxins belonging to the DON group. They found

DON-3-glucoside to be the most prevalent compound being transferred into beer, its levels exceeded those of free DON and its acetylated forms.

1.3.2 Milling

Cereal grains are often milled before further processing. Most mycotoxins tend to concentrate in the outer layer fractions of the grains, other parts (eg. white flour, maize grits) are usually contaminated with lower concentrations of mycotoxins than in the original whole grain (Magan & Olsen, 2004, p.227).

Lee et al. (1987) reported that after milling of wheat grains, the percentages remaining in flour fraction were about 30–80%, 60–70%, and 30–50% for nivalenol, DON, and zearalenone, respectively. In the out layer fractions (bran and shorts), the concentrations of these mycotoxins increased by about 2 folds compared to those in wheat grains.

In case of DON, more significant differences between the outer layer fractions and flour fraction have been reported. Simsek et al. (2012) found that the concentration of DON in flour was reduced by 61.8% from that in whole wheat grains, whereas the concentrations in bran or shorts were about 3 folds compared to those in whole wheat.

In a study of Argentinian corn (Broggi et al., 2002), the concentrations of fumonisins in the germ and bran fractions were found to be approximately 3 times higher than those in whole corn. In contrast to this, the concentrations in “C” grade flour were only 17–28% compared to the original levels in whole corn.

Scudamore et al. (2003) showed that compared to whole wheat grains, concentration of ochratoxin A increased in bran fraction, and decreased in white flour fraction.

1.3.3 Bread making

Among various cereal processing technologies, bread making has gained considerable attention as bread is the staple food in many regions all over the world.

Bread making usually involves fermentation of the dough and final baking, and the temperature and duration of both periods vary in different methods. Therefore, it is not unexpected that different results were obtained from different research groups. Such is the case with the *Fusarium* mycotoxin DON.

Until now, a number of researches have been focused on changes of DON during bread making. However, the results are highly inconsistent, ranging from no change (Lancova et al., 2008a) to a maximum decrease of 96.6% (Neira et al., 1997).

In the report by Lancova et al. (2008a), practically no changes of DON occurred comparing the bread baked at 240 ℃ for 14 min and the initial flour, a total fermentation period at 30 ℃ for 95 min was included in the whole bread making procedure. In contrast, a slight decrease of 13% between bread and flour was found by the same group using very similar conditions (240 ℃) (Kostelanska et al., 2011a).

Scudamore et al. (2009) also concluded that DON was stable after fermentation at 40–45 ℃ for a maximum of 60 min, followed by final baking at 210 ℃ for 21 min. The loss of DON was no more than 11% from flour to bread.

Higher reductions were reported by Pacin et al. (2010), who found that DON in French bread and Vienna bread were reduced by 42.3% and 58.3%, respectively, in average compared to flour. Unfortunately, the fermentation and baking conditions were not clear. Similarly, Voss & Snook (2010) found that 50% of the spiked DON was reduced from flour to bread, after 4 h of fermentation and 18 min of baking at 227 ℃.

Even more remarkably, a maximum reduction of 96.6% of DON was reported by Neira et al. (1997) between the bread and the unfermented dough. In their report, different procedures were used during bread production, which included a prolonged fermentation time between 5 and 11 h at 25 ℃, followed by final baking at 210 ℃ for 10–40 min. As a consequence, the decrease of DON also ranged widely from 16.8% to 96.6%. Although the authors mentioned a positive relationship between increase of baking time and the percentage of reduction, the detailed data were not given. Therefore, it is unclear under what specific condition the 96.6% reduction was reached. In any case, the decrease was extremely different compared to other reports.

Contrary to the varying decreases of DON mentioned above, Simsek et al. (2012) reported that DON was increased by 40.8% in the bread than in the unfermented dough. The baking procedure included fermentation at 30 ℃ for 180 min, followed by baking at 220 ℃ for 25 min. Further investigation of enzyme treatments revealed that DON increased significantly ($p < 0.05$) in the xylanase or protease

treated wheat, leading to the suggestion that DON may be bound to the cell wall matrix or protein component of wheat, and that baking may release the bound mycotoxin from the flour.

Studies on the change of other mycotoxins during bread making have also been reported, including aflatoxins, T-2 and HT-2 toxins, nivalenol, ochratoxin A, and ENNs (Amra et al., 1996; Monaci et al., 2011; Valle-Algarra et al., 2009; Vaclavikova et al., 2013).

The study by Amra et al. (1996) showed that about 86% of aflatoxins B1 and G1 were destructed during French bread making, which included fermentation at 30 ℃ for 3 h, and baking at 230 ℃ for 20 min.

Valle-Algarra et al. (2009) observed an average decrease of 32.9% of ochratoxin A after fermentation at 29–30 ℃ for 1 h, followed by baking at different temperature/time combinations (190 ℃/50 min, 207 ℃/35 min, and 240 ℃/30 min). In comparison, the trichothecenes nivalenol, 3-acetyl-DON, and DON were reduced by 76.9%, 65.6%, and 47.9%, respectively.

Monaci et al. (2011) found that 20–30% of HT-2 toxin and DON (artificially added in dough) were reduced after 30 min baking at 200 ℃, whereas most of T-2 toxin was converted to HT-2 toxin by enzymatic hydrolysis during dough preparation.

The change of ENNs during bread making was described in a recently published paper (Vaclavikova et al., 2013), where the ENNs B and B1 in bread dropped to 60% and 50%, respectively, compared to their original levels in wheat flour.

However, all the above mentioned bread making procedures adopted a straight dough baking technique, whereas no detailed reports have addresssed the changes of mycotoxins during sourdough bread making as far as I know. The latter differs from the usual straight dough baking in the use of sourdough, which is defined as the dough made from cereals, liquids, and microbes (such as lactic acid bacteria and yeast) in an active state (Flander et al., 2011). Sourdough fermentation can improve nutritional value or promote healthiness of cereal foods, and wholemeal sourdough baking of rye or wheat is a typical example (Katina et al., 2005). Until now, it is unclear whether the presence of active microorganisms during the prolonged (for example 24 h) sourdough preparation stage and subsequent bread dough fermentation might affect the levels of mycotoxins. Therefore, it would be interesting to study the changes of ENNs and BEA during sourdough bread making.

1.4 Occurrence of mycotoxins in herbal medicines

Throughout the history, virtually all human cultures have used a variety of plants or materials derived from plants for the prevention and treatment of disease (Matthews et al., 1999). In some countries such as China, the use of medicinal herbs can date back to thousands of years ago.

Today, according to World Health Organization, in some Asian and African countries, 80% of the population still depends on traditional medicine (mainly herbal medicines) for primary health care (WHO website, 2013). Herbal and other "alternative" treatments are also popular in the developed world, being used by about 33% of Americans and 50% of Australians (Shaw, 1998). In Western Europe, annual revenues of herbal treatments reached US$ 5 billion in 2003–2004 (WHO website, 2013).

However, like other plants, medicinal herbs could also be infected with mycotoxin-producing fungi during growth, processing, or storage if the conditions were favorable for the moulds. The safety of medicinal herbs is therefore receiving more and more attention.

In the recent decade, increasing researches of mycotoxins in medicinal herbs have been carried out. Among them, aflatoxins are the most popular due to their high toxicity. Determination of aflatoxins B1, B2, G1, and G2 in herbal medicines have been reported in countries inclduing Spain (Gómez-Catalán, et al., 2005), Malaysia, Indonesia (Ali et al., 2005), Italy (Romagnoli et al., 2007), and China (Han et al., 2010; Ip & Che, 2006). Methods for the detection of other mycotoxins in herbal medicines have also been reported, including zeralenone and its derivatives (Gray et al., 2004; Han et al., 2011), DON, nivalenol (Yue et al., 2010), fumonisins (Kong et al., 2012), and T-2 toxin (Yue et al., 2009). However, none was concerned with ENNs and BEA as far as I know.

1.5 Objectives

Although no maximum levels of ENNs and BEA have been legislated until now, their toxic effects in combination with high contamination levels in food suggest that they might pose possible harzards to human health. EFSA has requested scientific opinions on the risks to human and animal health related to ENNs and BEA in food and feed (EFSA website, 2010).

As no isotope labeled standards of ENNs and BEA are available, this study aims to synthesize $^{15}N_3$-labeled ENNs and BEA, develop SIDA of these mycotoxins, and apply the method in a series of samples.

The objectives of this study are as follows:

- To synthesize $^{15}N_3$-labeled ENNs and BEA by fungal biosyntheses, and to develop SIDA for ENNs and BEA in food samples.
- To follow the fate of ENNs and BEA during beer production (mainly including malting and brewing) by SIDA.
- To study the effects of milling and sourdough bread making on ENNs and BEA by SIDA, using wheat and rye grains as raw materials.
- To determine contamination levels of ENNs and BEA by SIDA in 60 Chinese medicinal herbs, which are widely used both as food and medicines in China.

2. Materials and methods

2.1 Materials

2.1.1 Equipments

2.1.1.1 HPLC-DAD

Table 2-1. Equipment for HPLC-DAD analysis

Item	Model	Manufacturer
Interface	D-7000	Merck Hitachi system, Tokyo, Japan
Autosampler	L-7200	- `` -
Pump	L-7100	- `` -
Detector	L-7455 diode array detector	- `` -
Column	Synergi Hydro-RP 80A, 250 mm × 3.0 mm i.d., 4 μm	Phenomenex, Torrance, CA, USA

2.1.1.2 LC-MS/MS

Table 2-2. Equipment for LC-MS/MS analysis

Item	Model	Manufacturer
Degasser	DGU-20A_3	Shimadzu USA manufacturing Inc., Canby, OR, USA
Liquid chromatography	LC-20AD SP	- `` -
Autosampler	SIL-20AC HT	- `` -
Communication bus module	CBM-20A	- `` -
Column oven	CTO-20AC	- `` -
Diode array detector	SPD-M20A	- `` -
Mass spectrometer	API 4000 QTrap	Applied Biosystems Inc., Foster City, CA, USA
Software	Analyst 1.5	- `` -

Switching valve	Rheodyne, K3186	IDEX Health & Science LLC, USA
Column 1	Synergi Polar RP 80A, 150 mm × 2.0 mm i.d., 4 µm	Phenomenex, Torrance, CA, USA
Column 2	YMC-Pack ProC18, 150 × 3.0 mm i.d., 3 µm	YMC Europe GmbH, Germany
Guard column	C-18, 4.0 × 2.0 mm i.d.	Phenomenex, Aschaffenburg, Germany

2.1.1.3 Other equipment

Table 2-3. Other equipment

Item	Model	Manufacturer
Rotary evaporator	Büchi Rotavapor R-200	Büchi, Switzerland
	Büchi RE121 Rotavapor	Büchi, Switzerland
Centrifuge	Jouan CR412	Jouan, France
UV-spectrometer	UV spectrometer Specord 50	Analytik Jena, Jena, Germany
Autoclave	SANOclav	Adolf Wolf, Germany
Ultrasonic bath	Sonorex Super RK 510 H	Bandelin, Berlin, Germany
Vortex	Genius 3	Ika, Staufen, Germany
Magnetic stirrer	RCT classic	Ika, Germany
Vacuum pump	Vacuubrand cvc3000	Vacuubrand, Germany
Shaker	VKS 75	Edmund Bühler, Tübingen, Germany
Mill 1	Universalmühler M20	Ika, Staufen, Germany
Mill 2	Quadrumat Junior	Brabender, Duisburg, Germany
Balance 1	Sartorius Basic BA210S	Sartorius, Germany
Balance 2	Bosch PE610	Bosch, Germany
Oven	Heraeus (0–240 °C)	Heraeus, Germany
Laboratory dishwasher	Miele professional 7883 CD	Miele, Germany

2.1.2 Chemicals

Potassium chloride, citric acid, iron(II) sulfate heptahydrate, ammonium sulfate, copper(II) sulfate pentahydrate, glucose, aluminium oxide (alumina), charcoal, and celite were purchased from Merck (Darmstadt, Germany).

Potassium dihydrogen phosphate, zinc sulfate monohydrate, manganese(II) sulfate monohydrate, sodium molybdate dihydrate, sodium nitrate, chlortetracycline hydrochloride, streptomycin sulfate, and agar were purchased from Sigma-Aldrich (Steinheim, Germany).

Acetonitrile and methanol were purchased from Merck (Darmstadt, Germany) and VWR Prolabo (Fontenay Sous Bois, France).

The following compounds were obtained from the sources given in parentheses:

magnesium sulfate heptahydrate (AppliChem, Darmstadt, Germany),

boric acid (Avantor Performance Materials, Deventer, Netherlands),

methanol-d_4 and chloroform-d_1 (Euriso-top, Gif sur Yvette Cedex, France),

^{15}N-sodium nitrate (98 atom% ^{15}N, Cambridge Isotope Laboratories, MA, USA),

BEA (AnaSpec, San Jose, USA),

ENN B (Bioaustralis, New South Wales, Australia),

ENNs A, A1, B1 (Enzo Life Sciences, Lörrach, Germany).

Water for HPLC was purified by a Milli-Q-system (Millipore GmbH, Schwalbach, Germany).

2.1.3 Consumables

Strata C-18-T SPE cartridges (55 µm, 140A, 1000 mg/6mL, Phenomenex, Torrance, CA, USA),

MycoSep 225 SPE cartridges (Romer Labs Inc., MO, USA),

597 ½ S&S folded paper filters (Schleicher & Schuell, Dassel, Germany),

0.45 µm membrane filters (Spartan 13/0.45 RC, Whatman, Dassel, Germany),

0.22 µm PVDF membrane filters (Zefa-Laborservice, Harthausen, Germany).

2.1.4 Culture media

2.1.4.1 Modified SNA medium

The modified SNA medium was modified based on SNA (Spezieller Nährstoffarmer Agar) medium (Leslie & Summerell, 2006, p.6).

KH_2PO_4	1 g
KNO_3	1 g
$MgSO_4 \cdot 7H_2O$	0.5 g
KCl	0.5 g
Glucose	0.4 g
Agar	20 g
Chlortetracycline	100 mg
Streptomycin	50 mg
Distilled water	to 1 L

2.1.4.2 Modified Czapek-Dox liquid minimal medium

The modified Czapek-Dox liquid minimal medium was modified based on Czapek-Dox minimal medium (Leslie & Summerell., 2006, p.10–11).

KH_2PO_4	1g
$MgSO_4 \cdot 7H_2O$	0.5 g
KCl	0.5 g
Glucose	30 g
$NaNO_3$	2 g
Trace element solution	0.2 ml
Distilled water	to 1 L

The trace element solution contains:

Citric acid	5 g
$ZnSO_4 \ 6H_2O$	5 g
$Fe(NH_4)_2(SO_4)_2 \ 6H_2O$	1 g
$CuSO_4 \ 5H_2O$	250 mg

$MnSO_4$	50mg
H_3BO_3 (Boric acid)	50 mg
Na_2MoO_4 $2H_2O$	50 mg
Distilled water	95 ml

2.1.4.3 Rice medium

Rice	25 g
Distilled water	15 mL/25 mL

2.1.5 Microorganisms

Fifty-seven *Fusarium* strains were screened for ENNs and BEA production, all the strains (**Table 2-4)** were provided by Prof. Dr. Ludwig Niessen (Chair of Technical Microbiology, Technische Universität München).

Table 2-4. 57 *Fusarium* strains screened for ENNs and BEA production

Species	Strain number (by TMW)	Strain number (by others)
F. proliferatum	4.0236	DSM 62261
F. proliferatum	4.0227	LN 32.90
F. proliferatum	4.0827	BBA 69540
F. proliferatum	4.0828	BBA 69541
F. proliferatum	4.0840	BBA 69589
F. proliferatum	4.0948	BBA 63624
F. proliferatum	4.0954	BBA 63634
F. proliferatum	4.0984	BBA 65640
F. proliferatum	4.1012	BBA 69011
F. proliferatum	4.1014	BBA 69013
F. proliferatum	4.1017	BBA 69070
F. proliferatum	4.1018	BBA 69071
F. proliferatum	4.2098	
F. proliferatum	4.2109	
F. oxysporum	4.0217	LN 359
F. oxysporum	4.0473	INRA 92009

F. oxysporum	4.0629	LN 76
F. oxysporum	4.0136	LN 368
F. oxysporum	4.0116	LN 331
F. oxysporum	4.0148	LN 190
F. oxysporum	4.1358	
F. oxysporum	4.0226	E 454
F. oxysporum	4.0221	DSM 62291
F. oxysporum	4.1744	
F. oxysporum	4.2167	
F. oxysporum	4.0471	INRA Foln8
F. oxysporum	4.0470	INRA Foln3
F. oxysporum	4.0225	DSM 2018
F. oxysporum	4.0210	DSM 62287
F. oxysporum	4.0228	
F. oxysporum	4.0114	LN 292
F. oxysporum	4.0474	INRA 92001
F. oxysporum	4.0472	INRA Foln24
F. oxysporum	4.1889	
F. oxysporum	4.0163	DSM 62292
F. oxysporum	4.2169	
F. oxysporum	4.2168	
F. oxysporum	4.2090	
F. sambucinum	4.0278	LN 43.89
F. sambucinum	4.0313	LN 47.89
F. sambucinum	4.0181	LN 152.89
F. sambucinum	4.0259	LN 24.90
F. sambucinum	4.0304	LN 377
F. sambucinum	4.0264	LN 150.89
F. sambucinum	4.0321	DSM 62186
F. sambucinum	4.0165	CBS 185.29
F. sambucinum	4.0796	BBA 62434
F. sambucinum	4.0917	BBA 64226

F. sambucinum	4.0962	BBA 64262
F. sambucinum	4.0976	BBA 64993
F. sambucinum	4.0977	BBA 94995
F. sambucinum	4.0978	BBA 64996
F. sambucinum	4.0979	BBA 64998
F. fujikuroi	4.0336	DSM 63217
F. fujikuroi	4.0628	LN 3.96
F. fujikuroi	4.0860	BBA 69741
F. fujikuroi	4.0953	BBA 63630

TMW = Technische Mikrobiologie Weihenstephan, Freising, Germany.

DSM = Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.

LN = Ludwig Niessen, Chair of Technical Microbiology, Technische Universität München, Germany.

BBA = Biologische Bundesanstalt für Land- und Forstwirtschaft, Berlin, Germany

E = Collection of Dr. G. Engelhard, Landesanstalt für Ernaehrung, Munich, Germany

INRA = Strain collection of Institut National de la Recherche Agronomique, Paris, France

CBS = Centraalbureau voor Schimmelcultures, the Netherlands

2.1.6 Raw materials for beer making

Table 2-5. Raw materials for beer making

Material	Description	Manufacturer/Provider
Spring barley*	Variety Quench	Syngenta Seeds, Bad Salzufflen, Germany
Yeast	*Saccharomyces cerevisiae* W 34/70	Hofbräuhaus Freising, Germany
Hop	Hallertau Hallertauer Select hop (5.1% alpha acids)	Simon H. Steiner GmbH, Mainburg, Germany

*Three batches of barley were used, batches QFc and QFa were artificially inoculated in field with *Fusarium culmorum* and *F. avenaceum*, respectively, and batch QC was the control group which remained un-inoculated. The inoculation was carried out by Chair of Phytopathology, Technische Universität München (Hu et al., 2014).

2.1.7 Raw materials for sourdough bread making

Table 2-6. Raw materials for sourdough bread making

Material	Description	Manufacturer
Wheat	Variety Pamier	
Winter ye	Variety Nikita	KWS Lochow GmbH
Sourdough starter	Böcker F20	Ernst Böcker GmbH, Minden, Germany

2.1.8 Chinese medicinal herbs

A total of 60 types of dried Chinese medicinal herbs were examined for contamination with ENNs and BEA. The samples (approximately 100 g for each type) were randomly purchased from traditional Chinese medicine stores in Zhejiang province, China. Their common names and pharmaceutical names were listed in **Table 2-7**.

Table 2-7. 60 Chinese medicinal herbs analyzed for occurrence of ENNs and BEA

Common name	**Pharmaceutical name**
Star anise	Fructus anisistellati
Hyacinth bean	Semen lablab album
Dahurian angelica root	Radix angelicae dahuricae
Ginkgo seed	Semen ginkgo
Lily bulb	Bulbus Lilii
Mint leaf	Herba menthae
Adzuki bean	Semen phaseoli
Colve	Flos caryophylli
Jack bean	Semen canavaliae
Finger citron	Fructus citri sarcodactylis
Fu-ling (Indian bread)	Sclerotium poriae cocos
Chinese raspberry	Fructus rubi
Licorice root	Radix glycyrrhizae
Goji berry/Wolfberry	Fructus lycii
Kudzuvine root	Radix puerariae

Pricklyash peel	Pericarpium zanthoxyli
Lotus leaf	Folium nelumbinis
Black sesame	Semen sesami nigrum
Black pepper	Fructus piperis nigri
Hemp fruit	Fructus cannabis
Sophora flower	Flos sophorae
Wrinkled giant hyssop herb	Herba agastaches
Sicklepod	Semen cassiae
Honeysuckle flower	Flos lonicerae
Ginger	Rhizoma zingiberis recens
Chrysanthemum flower	Flos chrysanthemi
Tangerine peel	Pericarpium citri reticulatae
Red tangerine peel	Exocarpium citri rubrum
Platycodon root	Radix platycodi
Kombu	Thallus laminariae
Longan pulp	Arillus longan
Luohanguo	Fructus momordicae
Lotus seed	Semen nelumbinis
Common purslane	Herba portulacae
Chinese quince fruit	Fructus chaenomelis
Boat-fruited sterculia seed	Semen sterculiae lychnophorae
Dandelion herb	Herba taraxaci
Gordon euryale seed	Semen euryales
Nutmeg	Semen myristicae
Cassia bark	Cortex cinnamomi
Chinese yam	Radix dioscoreae oppositae
Hawthorn fruit	Fructus crataegi
Seabuckthorn berry	Fructus hippophae
Mulberry leaf	Folium mori
Mulberry fruit	Fructus mori
Spina date seed	Semen ziziphi spinosae
Peach kernel	Semen persicae

Smoked plum	Fructus mume
Fennel	Fructus foeniculi
Bitter apricot seed	Semen armeniacae amarum
Cogongrass rhizome	Rhizoma imperatae
Reed rhizome	Rhizoma phragmitis
Longstamen onion bulb	Bulbus allii macrostemi
Bitter cardamom	Fructus alpiniae oxyphyllae
Heartleaf houttuynia herb	Herba houttuyniae
Coix seed	Semen coicis
Chinese date	Fructus zizyphi
Cape jasmine fruit	Fructus gardeniae
Perilla leaf	Folium perillae
Perilla seed	Semen perillae

2.2 Methods

2.2.1 Screening of ENNs and BEA-producing *Fusarium* strains

2.2.1.1 Fungal culture on different media

Modified SNA medium

The modified SNA medium was used for maintenance of the *Fusarium* strains. SNA is a weak nutrient agar, culture degeneration which is common on many other synthetic media, usually does not occur on SNA (Leslie & Summerell, 2006, p.6). Each strain was cultivated in a 9 cm petri dish containing 20 mL of the modified SNA medium.

Rice media

Six possible ENNs- and/or BEA-producing *Fusarium* strains (*F. proliferatum* 4.0227, *F. proliferatum* 4.2098, *F. sambucinum* 4.0278, *F. sambucinum* 4.0304, *F. fujikuroi* 4.0628, and *F. oxysporum* 4.0473) were randomly selected to test the effect of two types of rice media, which contained 60% and 100% additional water, respectively.

In each Erlenmeyer flask with vented cap were kept 25 g rice and 15 mL/25 mL water, the flasks were autoclaved at 121 ℃ for 25 min, and, after cooling down to room temperature, the fungi previously grown on modified SNA medium were transferred to rice media.

After one month growth at room temperature, the rice culture was dried in an oven at 60 ℃ for 4 h and ground to fine powder.

The rest of the *Fusarium* strains listed in **Table 2-4** were cultivated on rice medium containing 60% of additional water.

Modified Czapek-Dox liquid minimal medium

Seven *Fusarium* strains cultivated on rice medium were found to be relatively potent ENNs/BEA-producers, they were further cultivated in the modified Czapek-Dox liquid minimal medium.

Each 250 mL Erlenmeyer flask was filled with 100 mL of the culture medium, autoclaved at 121 ℃ for 25 min before inoculation of the fungi, after cultivation for one week, the cultures were harvested.

To find out the influence of $(NH_4)_2SO_4$ and $NaNO_3$ on the production of ENNs and BEA, strains *F. sambucinum* 4.0979 (ENNs-producer) and *F. fujikuroi* 4.0860 (BEA-producer) were cultivated in the modified Czapek-Dox liquid minimal media without $(NH_4)_2SO_4$ and with different amounts of $NaNO_3$, namely 1 g/L, 2 g/L, and 3 g/L of $NaNO_3$. Normal Czapek-Dox liquid minimal medium (containing trace element of $(NH_4)_2SO_4$ and 2 g/L of $NaNO_3$) was used as control. The trial was carried out in triplicate.

2.2.1.2 Extraction of ENNs and BEA from fungal culture

Rice media

For the 6 randomly selected *Fusarium* strains used to test the effect of two types of rice media, 2 g of the ground rice culture was extracted with 20 mL of ACN-H_2O (84:16, v/v), stirred with a magnetic stirrer for 4 h, and standed still for 2 d.

Two sample preparation methods were compared:

(a) Cleanup with alumina-charcoal-celite (5:7:3) cartridge

An aliquot of 2 mL of the extract was passed through a self-made alumina-charcoal-celite (5:7:3) cartridge (Trucksess et al., 1987), the eluate was dried with a rotary evaporator, the residue was reconstituted to 240 µL ACN-H_2O (5:1, v/v).

(b) No cleanup, i.e. "dilute-and-shoot"

An aliquot of 2 mL of the extract was diluted by 5 folds without further SPE cleanup.

Each sample was filtered through a 0.22 µm membrane filter before HPLC injection.

For the rest of *Fusarium* strains in **Table 2-4**, 2 g of the ground rice culture was extracted with 20 mL of ACN-H_2O (84:16, v/v), and the dilute-and-shoot sample preparation method was adopted, no further cleanup was applied to the extract except filtration through 0.22 µm membrane filter before HPLC analysis.

Modified Czapek-Dox liquid minimal medium

An aliquot of 40 mL of the culture broth including most of the mycelia was centrifuged at 4000 rpm for 10 min, the supernatant was discarded, the residue, i.e. the mycelia, was dried in an oven at 60 ℃ for 18 h. Subsequently, 10 mL of ACN-H_2O (84:16, v/v) were added to the dried mycelia, which were extracted in an ultrasonic bath for 15 min, then standed still for 2 d.

2.2.2 Biosynthesis of $^{15}N_3$-labeled ENNs and BEA

2.2.2.1 Fungal culture

Czapek-Dox liquid minimal medium, with the normal $NaNO_3$ replaced by $Na^{15}NO_3$, sucrose replaced by glucose, and $(NH_4)_2SO_4$ eliminated, was used as culture medium. Five 250 mL Erlenmeyer flasks, each containing 100 mL of the modified Czapek-Dox liquid minimal medium were autoclaved at 121 ℃ for 25 min. An ENNs-producer, *Fusarium sambucinum* strain 4.0979 previously grown on modified SNA medium was transferred to the five flasks, and incubated on a shaker (128 rpm) at 25 ℃ for 7 d. A BEA-producer, *Fusarium fujikuroi* strain 4.0860 was cultured likewise to produce BEA.

2.2.2.2 Extraction of $^{15}N_3$-labeled ENNs and BEA

The culture broth of the ENNs/BEA producer was centrifuged at 4000 rpm for 10 min, and the supernatant was discarded as the content of ENNs/BEA was negligible. The residue, i.e. the harvested mycelia, was dried in an oven at 50 ℃ for 18 h, and extracted with 100 mL of ACN-H_2O (84:16, v/v) in an ultrasonic bath for 3 × 15 min, followed by extraction on a shaker for 2 d. The crude extract was filtered through folded papers. The filtrate was then processed according to Song et al. (2008) with minor modifications. Namely, the filtrate was defatted twice with 200 mL of hexane, and the bottom layer was evaporated to dryness, the residue was dissolved in 200 mL of MeOH-H_2O (55:45, v/v) and extracted twice with 200 mL of CH_2Cl_2. Then, the CH_2Cl_2 phase was evaporated and the residue was dissolved in 5 mL of MeOH. This solution was passed through Strata C-18-T SPE cartridges. The cartridges were eluted with MeOH, the eluate was concentrated to 2 mL, and filtered through a 0.45 μm membrane filter prior to HPLC injection.

2.2.2.3 Preparation of $^{15}N_3$-labeled ENNs and BEA by HPLC

HPLC-DAD analyses and preparations were performed using an analytical Merck Hitachi system, with a Synergi Hydro-RP 80A column. HPLC conditions were set up using a constant flow of 0.6 mL/min, injection volume was 15 μL.

For the isolation of ENNs, two HPLC methods using different mobile phase conditions were tested during the first round of HPLC.

HPLC method (a): mobile phase MeOH-H_2O:

The starting mobile phase was 78% MeOH, kept for 5 min, linearly raised to 92% MeOH in 20 min, and maintained for 1 min before returning to the starting condition in 4 min.

HPLC method (b): mobile phase ACN-H_2O:

Method (b) included a very shallow gradient elution, which started with 65% ACN, kept for 5 min, linearly increased to 68% ACN in 10 min, and maintained for 12 min before it was switched back to the starting condition in 3 min.

The ENNs were detected at a wavelength of 208 nm (later 203 nm due to the shift of the DAD detector). Each major peak from the two HPLC methods was collected, and measured with LC-MS/MS to check its composition of ENNs. According to LC-MS/MS results, method (b) was chosen, from which 4 fractions each mainly containing one of the ENNs were collected. The HPLC separation was then repeated and the five fractions were collected and pooled. Each pooled fraction was evaporated to dryness under reduced pressure and redissolved in 180 µL of MeOH.

To further purify the five fractions, a second round of HPLC separation using the same system was performed for each of them separately. The flow was kept constant at 0.6 mL/min, MeOH-H_2O (70–80% MeOH for different fractions) was used as starting eluent, maintained for 5 min before rising to 92% MeOH over 20 min, then kept for 1 min, and taken back to the starting ratio in 4 min.

BEA was isolated from the extract with similar procedures, i.e. one major peak containing BEA was collected from the first round of HPLC, and the fraction was further purified with a second round of HPLC.

Each rechromatographed fraction was coinjected with pure standard for confirmation, and their purity was further verified by LC-MS in the full scan mode as described below.

2.2.2.4 LC-MS and LC-MS/MS measurement

Liquid chromatography was carried out on a Shimadzu LC-20A Prominence system with a Synergi Polar RP 80A column.

The starting mobile phase ACN-H_2O (65:35, v/v) was kept constant for 5 min and linearly raised to 75% ACN in 7 min. After 1 min at 75% ACN, the gradient was increased to 100% ACN in 2 min, and held for 1 min before returning to the starting condition in 3 min. Injection volume was 10 μL, flow rate was 0.2 mL/min, and equilibration time between two runs was 5 min. Data acquisition was carried out using Analyst 1.5 software.

The LC system was interfaced to a mass spectrometer operated in the positive ESI mode.

The ion source parameters were set as follows: curtain gas, 10 psi; temperature, 450 ℃; ion source gas 1, 45 psi; ion source gas 2, 50 psi; ion spray voltage, 5500 V.

MS parameters were optimized by direct infusion of each standard solution (40 ng/mL) into the source using automatic and manual tuning. These parameters were listed in **Table 2-8**.

Table 2-8. LC-MS/MS parameters including transition ions, declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP)

		transition ion (*m/z*)				
compound	ion type[a]	unlabeled	labeled	DP	CE (V)	CXP
ENN A	quantifier	682→210	685→211	121.0	39.0	10.0
		682→228	685→229	121.0	39.0	12.0
ENN A1	quantifier	668→210	671→197	131.0	39.0	12.0
		668→196	671→211	131.0	33.0	12.0
		668→228	671→229	131.0	39.0	12.0
ENN B	quantifier	640→196	643→197	121.0	37.0	10.0
		640→214	643→215	121.0	37.0	10.0
ENN B1	quantifier	654→196	657→197	131.0	41.0	10.0
		654→210	657→211	131.0	35.0	10.0
		654→214	657→215	131.0	37.0	10.0
BEA	quantifier	784→244	787→245	126.0	41.0	14.0
		784→262	787→263	126.0	35.0	14.0

[a]transition ions other than quantifiers are qualifiers.

Full scan spectra for confirmation of the purified compounds were recorded in a mass range from *m/z* 200 to 1500 and a scan time of 1.0 s. For MS/MS measurements, the mass spectrometer was operated in the MRM mode, a valve was used to divert the column effluent to the mass spectrometer from 5 to 13.5 min and to waste for the rest of the run.

2.2.2.5 ^{1}H-NMR

The structures of purified compounds were characterized by ^{1}H-NMR on a Bruker AV III system (Bruker Rheinstetten, Germany) operating at a frequency of 500.13 MHz. All five compounds were dissolved in $CDCl_3$.

2.2.2.6 Quantitation of $^{15}N_3$-labeled ENNs and BEA by qNMR

The method of qNMR for quantitation of $^{15}N_3$-labeled BEA, ENN A and ENN A1 was similar to that described by Korn et al. (2011). Briefly, the purified compounds were dissolved in 600 µL of methanol-d_4, and analyzed in 5 × 178 mm NMR tubes (Norell, ST500-7, Landisville, USA). A caffeine sample of known concentration was used as external standard. For quantitation, the signals at 7.87 ppm (caffeine), 5.47 ppm ($[^{15}N]_3$-BEA), 5.14 ppm ($[^{15}N]_3$-ENN A), and 5.09 ppm ($[^{15}N)_3$-ENN A1) were chosen. Intensity of the signal was integrated manually.

2.2.2.7 Preparation of standard solutions

All standard solutions were prepared in MeOH. The concentrations of labeled ENN A, ENN A1 and BEA were determined by qNMR described above, stock solutions of 100 µg/mL and 10 µg/mL were prepared. The UV absorptions of the 10 µg/mL ENN A and ENN A1 were determined on a UV spectrometer at the maximum absorption wavelength of 203 nm in triplicates. The ratio between the molar extinction coefficients of ENN A and ENN A1 was 1.007, which confirmed the assumption that the molar extinction coefficients of ENNs A, A1, B, and B1 are all the same since they differ only in the side chains which are devoid of UV chromophores. Based on this notion, the concentrations of ENN B and ENN B1 were determined by comparing their UV absorptions at 203 nm to those of ENN A and ENN A1. Stock solutions of 100 µg/mL were prepared for labeled ENN B and ENN B1, as well as all the unlabeled ENNs and BEA. All solutions were stored in the dark at 4 ℃.

2.2.3 Analysis of ENNs and BEA in cereals and related food by SIDAs

2.2.3.1 Sample preparation

All the cereals and related food samples were purchased from local markets in Germany, except barley malts, which were obtained from Bavarian malt producers. The samples were ground into fine powder before extraction. For each dried sample, 1 g was weighed and spiked with 10 ng (100 µL of a 100 ng/mL solution in ACN) of each of the labeled standards, after the solvent was evaporated, the sample was suspended in 10 mL of ACN-H_2O (84:16, v/v), vortexed for 1 min and extracted for 1.5 h, after which each sample was centrifuged at 4000 rpm for 10 min, and 1 mL of the supernatant was filtered through a 0.45 µm membrane filter prior to HPLC injection.

To test the performance of MycoSep 225 SPE cartridge, a wheat sample was extracted as described above, the supernatant was then treated in the following two ways: (a) no SPE cleanup, only filtered through a 0.45 µm membrane filter; (b) passed through MycoSep 225 SPE cartridge, and 1 mL of the eluate was concentrated to 100 µL, filtered through a 0.45 µm filter before HPLC. Both samples were measured by HPLC-MS/MS and HPLC-DAD. The results showed that MycoSep 225 treatment did not lead to better performance, therefore no SPE cleanup was used in further experiments.

2.2.3.2 Method validation

Calibration and quantitation

Constant amounts (10 ng) of labeled standard (S) were mixed with varying amounts of analyte (A) in molar ratios between 0.1 to 10 (1:10, 1:5, 1:2, 1:1, 2:1, 5:1, 10:1). After LC-MS/MS measurement, response curves were obtained from molar ratios [n(A)/n(S)] versus peak area ratios [A/(A)/A(S)], and response functions were obtained using linear regression, the linearity was checked by Mandel test. The contents of ENNs and BEA in samples were calculated using the respective response function.

LODs and LOQs

LODs and LOQs were calculated according to the procedures suggested by Vogelgesang and Hädrich (1998). A potato starch free of ENNs and BEA was used as blank sample, which was spiked with ENNs and BEA at four different amounts (5, 20, 35, and 50 µg/kg), each in triplicate. The samples were extracted and analyzed as described before.

Precision

Intra-day (n = 5) and inter-day (n = 3) precision was determined within 6 weeks. As no single sample that contained all four ENNs as well as BEA was found by that time, precision was determined with three samples: a naturally contaminated whole wheat flour sample was used to measure ENNs A1, B and B1, a naturally contaminated wheat grain sample was used for ENN A, and a rice sample that contained none of these mycotoxins was spiked with 45 µg/kg of BEA for determination as no naturally contaminated BEA sample was available.

Recovery

Blank samples (potato starch) were spiked in triplicate with different amounts (20, 35, and 50 µg/kg) of ENNs and BEA, and analyzed as described before. Recovery was calculated as the mean of the spiking experiments.

2.2.4 Fate of ENNs and BEA during beer making

2.2.4.1 Malting process

The malting process was performed according to the standard MEBAK procedure (Anger, 2006). Each batch of barley grains was subjected to 2 steps of steeping to reach a moisture content of 55%. The first steeping took 5 h by immersing the barley grains in 8 L of water at 15 ℃, afterwards the water was drained, and the barley grains were kept in the germination room until the next day for the second steeping, which took 4 h under the same condition. Subsequent germination was carried out at 14.5 ℃ for 5 d in humidified air. The germinated barley grains, i.e. the green malt, were kilned at 50 ℃ for 16 h, then at 60 ℃ for 1 h, at 70 ℃ for 1h, and finally at 80 ℃ for 5 h. After kilning, the brittle rootlets were removed from the kilned malt.

The key steps of malting and brewing processes were shown in **Figure 2-1**.

Table 2-9 presents the weight or volume of the materials during each step of malting and brewing.

2.2.4.2 Brewing process

Wort production was carried out in a 10 L scale pilot brewing plant. For each batch, 1.2 kg of the kilned malt was ground into particles and mixed with 5 L of water for mashing, during which period the water was first kept at 62 ℃ for 30 min, then at 72 ℃ for another 30 min, and finally at 76 ℃ for 5 min. The mixture was then separated into sweet wort and spent grains by lautering, i.e. the wort was filtered through the layer formed by grain particles. In the next step, about 8 g of hop was added to the sweet wort and boiled until the wort reached 11.5 °P (degree Plato, specific gravity of the extract, equivalent to grams of sucrose in 100 g solution at 20 ℃). After cooling and precipitation, the trub was separated from the hopped wort, and 70 g of yeast was added to the latter, the subsequent 6 d fermentation tookplace at 12 ℃. At the end of fermentation, the brewing tanks containing the green beer were kept at 16 ℃ for 3 d, followed by 10 d at 0 ℃ for maturation. After maturation, the beer was filtered through a filter sheet SEITZ-KS 80 (Pall Filtersystems GmbH, Bad Kreuznach, Germany) before bottling.

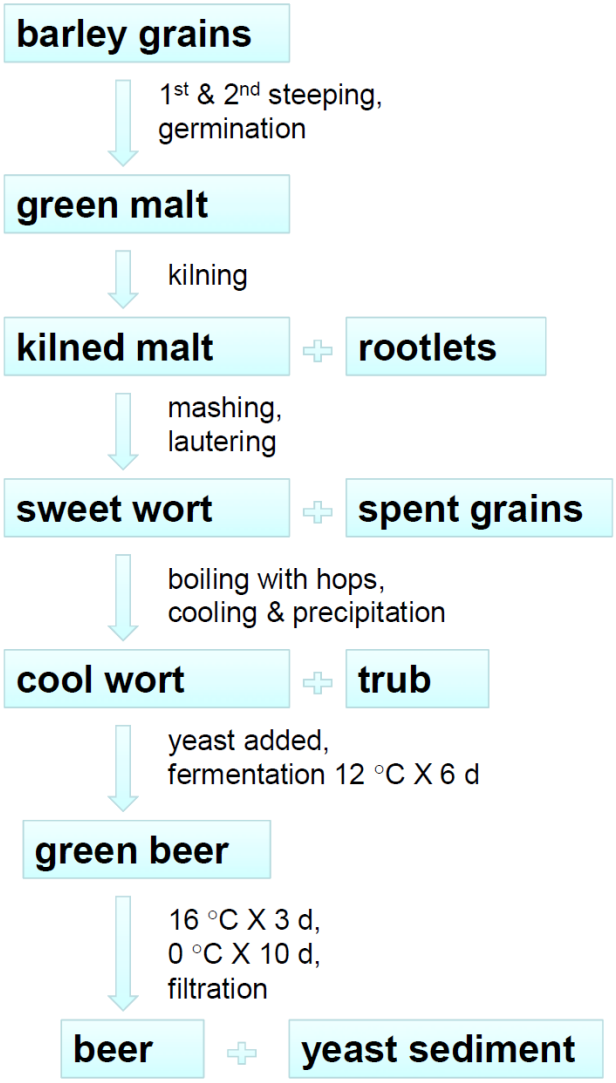


Figure 2-1. Scheme of key steps of malting and brewing processes

Table 2-9. Weight/volume of materials during each step of beer making

	QC	QFc	QFa
barley grains	1730 g	1707 g	1746 g
1st steeping: 5 h	6 L water	8 L water	8 L water
2nd steeping: 4 h	8 L water	8 L water	8 L water
kilned malt	1402 g	1326 g	1351 g
rootlets	62.7 g	87.5 g	91.0 g
sweet wort	8821 g	8617 g	8714 g
spent grains	418.1 g	480.5 g	410.3 g
hop	8.5 g	8.1 g	7.85 g
cool wort	6919 g	6478 g	6172 g
trub (dry)	38 g	53 g	56 g

2.2.4.3 Sampling

Samples were taken during each key step of the malting and brewing processes (**Figure 2-1**), including barley grains, first and second steeping water, green malt, kilned malt, rootlets, sweet wort, spent grains, cool wort, and trub. During the fermentation period, samples were taken every day. In addition, samples were taken after the three-day maturation at 16 ℃, as well as after the ten-day maturation at 0 ℃ of the green beer. Filtered beer, yeast sediment, and hop were also analyzed.

2.2.4.4 Extraction of ENNs and BEA

As the green malt samples were too wet to be handled, and as the wetness might lead to inhomogeneity of the samples, the green malt samples were sterilized with 70% ethanol and dried at room temperature for 2 d before being ground and extracted. The moisture contents of barley grains and the dried green malt are given in **Table 2-10**. The rest of solid samples were ground and homogenized before extraction. The liquid samples were used directly. The three trub samples, which were separated from the boiled wort by precipitation, were dried at 80 ℃ in an oven for 12 h before extraction, as they contained variable contents of liquid. For each sample, 1 g was spiked with 10 ng (100 µL of a 100 ng/mL solution in ACN) of each of the labeled standards, the sample was suspended in 10 mL of ACN-H_2O (84:16, v/v), vortexed for 1 min and extracted by shaking for 4 h, after which the sample was centrifuged at 4000 rpm for 10 min, and 1 mL of the supernatant was filtered through a 0.45 µm membrane filter prior to HPLC. For the samples which fell out of the linear range of the calibration curves, a second analysis was carried out, the extraction procedure was repeated, but the labeled standards were not added in the beginning. Instead, depending on the contamination levels, 0.1 mL of the extract was blended with 10 or 100 ng of each standard after extraction. Completeness of extraction and equilibration with the internal standards was verified by a comparison experiment using a barley sample (QFc). Before extraction, 20 ng of labeled ENN A1 and 200 ng of labeled ENN B1 were added to 1 g of the barley sample. In comparison, another 1 g of the same sample was extracted without addition of labeled standards, after extraction, 0.1 mL of the latter extract was blended with 10 ng of labeled ENN A1 and 10 ng of labeled ENN B1.

Table 2-10. Moisture contents of barley grains and dried green malt

	QC	QFc	QFa
barley grains	10.33%	10.04%	10.16%
green malt	9.11%	9.14%	11.77%

2.2.4.5 Method validation

The method validation was updated as a different type of HPLC column was used. It was performed analogously to that mentioned in section 2.2.3.2. For the determination of LODs, LOQs, as well as for recoveries, a blank potato starch was spiked with ENNs and BEA at four different levels (2, 5, 15, and 20 µg/kg), each in triplicate. Intraday (n = 5) and interday precision (n = 3) were determined using the barley batch QC.

2.2.4.6 Analysis of ENNs and BEA

Liquid chromatography was carried out on a Shimadzu LC-20A Prominence system using a YMC-Pack ProC18 column coupled to a Phenomenex C-18 guard column. The starting mobile phase ACN-H_2O (80:20, v/v) was kept constant for 5 min, then linearly raised to 100% ACN in 10 min, and held for 3 min before returning to the starting conditions. The injection volume was 10 µL, flow rate was 0.2 mL/min, and equilibration time between two runs was 5 min. Data acquisition and processing were carried out using Analyst 1.5 software.

For routine measurement, the LC was interfaced to a mass spectrometer operated in the positive ESI and MRM mode. MS parameters were identical with those mentioned in section 2.2.2.4. The effluent from the column was directed to the mass spectrometer from 11 to 21 min and to the waste for the rest of the run using a switching valve.

To check whether the sample extract would pose a potential hazard to the mass spectrometer, a kilned malt sample was measured on the LC-MS/MS system combined with a Shimadzu PDA (photodiode array) detector. A Shimadzu companion software was used in addition to Analyst 1.5 software for data acquisition.

2.2.4.7 Data analysis

All determinations were made in triplicate. Concentrations of ENNs and BEA presented in **Tables 3-16 to 3-18** were means of the three determinations. The concentrations were given on an "as is" basis, i.e. without correction for moisture contents. The total amount of green malt of each batch was corrected based on moisture content of barley grains. The total amounts of ENNs and BEA in barley grains, green malt, and kilned malt were analyzed by one-way analysis of variance (ANOVA) using PASW Statistics 18.0 (SPSS Inc., Chicago, USA) at $p < 0.05$.

2.2.5 Effect of sourdough bread making on ENNs and BEA

2.2.5.1 Milling

Wheat grains of the variety Pamier, and winter rye grains of the variety Nikita, both naturally contaminated with ENN B and ENN B1, were used as raw materials for the study.

The unconditioned whole grains of wheat and rye were milled using a laboratory mill (Quadrumat Junior) to obtain two fractions, i.e. flour and bran. The bran fraction was further ground to fine particles with a coffee grinder (Bosch). The mass ratio between wheat flour and wheat bran was approximately 77:23, and that between rye flour and rye bran was approximately 50:50. In the subsequent procedures, wheat flour was used for wheat bread making. For rye bread making, a 1:1 mixture of rye flour and rye bran (referred to as wholegrain rye flour in the following text) was used in order to reach relatively higher contamination levels of ENNs in the starting material.

The moisture contents of the grains and flours were determined by drying 100 g of each sample in an oven at 110 ℃ for 23 h. The wheat and rye grains had 10.5% and 13.7% moisture, respectively. The wheat flour had a moisture content of 10.7%, and the wholegrain rye flour had a moisture content of 14.0%.

2.2.5.2 Micro-scale sourdough preparation

As it is difficult to get a thoroughly homogenized sourdough mixture for the determination of ENNs and BEA, a micro-scale sourdough preparation procedure was carried out to determine its influence on the concentration of these mycotoxins. For each sample, 0.9 g of a 1:1 mixture of wheat and rye flour was blended with 0.1 g of sourdough starter, and 1.5 mL of water spiked with BEA was added. After vortexing for 1.5 min, the samples were divided into 2 groups, each containing 3 parallel samples, one group was kept in an oven at 30 ℃, and the other at 40 ℃ and both were fermented for 24 h. Inside the oven, filter paper was sprayed with water and partly soaked in water to reach a relative humidity (rh) of approximately 100%. All the 100% rh's mentioned below were achieved in the same way. A control group was prepared in triplicate shortly before extraction of ENNs and BEA.

2.2.5.3 Sourdough bread making

For the preparation of sourdough, 27 g of flour (wheat flour or wholegrain rye flour in case of wheat or rye sourdough, respectively), 3 g of sourdough starter, and 45 mL of water spiked with 13 µg of BEA were mixed to obtain a dough yield of 250. For the spiking procedure, BEA first was dissolved in ACN, then the solution was transferred to a beaker, and ACN was evaporated under gentle air. Subsequently, water was added followed by gently shaking and, after a while, the solution was transferred to a flask and used for sourdough preparation. The sourdough mixture was fermented in an oven at 30 °C, 100% rh for 24 h before being used for sourdough bread making.

Wheat sourdough bread was prepared with the following ingredients: wheat flour (120 g), fermented wheat sourdough (75 g), water (35 mL), sugar (1.5 g), salt (3 g), fat (1.5 g), and pressed baker's yeast (6 g). The ingredients were mixed thoroughly and kneaded with a hand mixer (myMix 300 W, Siemens, Munich, Germany), the dough was rested at 30 °C, 100% rh for 10 min, then divided into 40 g pieces, molded by hand, and proofed at 30 °C or 50 °C, 100% rh for 45 min. Finally, the dough proofed at 30 °C was baked at 200 ± 6 °C for 25 min. The whole sourdough bread making process including sourdough preparation is shown in **Figure 2-2**.

Rye sourdough bread was prepared with similar ingredients except that wholegrain rye flour was used instead of wheat flour, and that the sourdough was prepared from wholegrain rye flour.

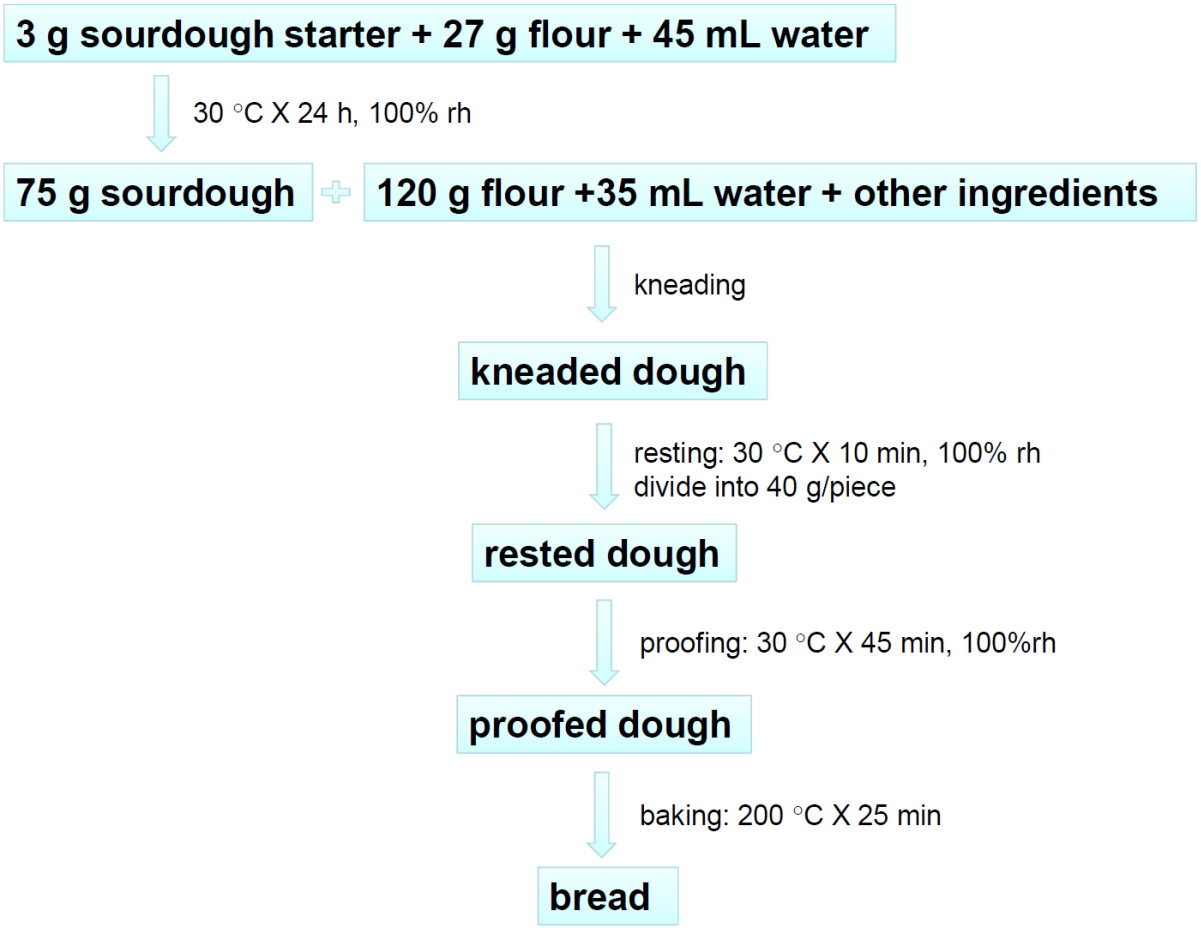


Figure 2-2. Scheme of sourdough bread making process

2.2.5.4 Sampling and extraction of ENNs and BEA

Analytical samples were taken in triplicate from each step of the milling and baking processes, including bran, flour, kneaded dough, rested dough, proofed dough, and crust and crumb of the final bread. Sourdough starter was also analyzed.

For the bran, flour, or sourdough starter, 1 g of each sample was spiked with 10 ng (100 µL of a 100 ng/mL solution in ACN) of $[^{15}N]_3$-ENN B, $[^{15}N]_3$-ENN B1, and $[^{15}N]_3$-BEA, and extracted with 10 mL of ACN-H_2O (84:16, v/v). For the various doughs, 1.6 g (equivalent to approximately 1 g of flour) of each sample was weighed and spiked with 10 ng of the isotope labeled internal standards, and extracted with 10 mL of ACN-H_2O (84:16, v/v) (water content of the dough was taken into account). The crust and crumb of bread were ground, and 1 g of each sample was weighed followed by the same spiking and extraction method to that of dough samples. Each sample from the micro-scale sourdough preparation procedure was spiked likewise and added with 8.5 mL of ACN for extraction. All the

samples were blended thoroughly, vortexed for 1.5 min, and extracted overnight. From each sample, 1 mL of the supernatant was filtered through a 0.22 µm membrane filter prior to LC-MS/MS injection.

2.2.5.5 Analysis of ENNs and BEA

LC-MS/MS measurements of ENNs and BEA were carried out on a Shimadzu LC-20A Prominence system interfaced to a mass spectrometer. A YMC-Pack ProC18 column with a Phenomenex C-18 guard column was used for compound separation. The LC-MS/MS conditions were identical with those mentioned in section 2.2.4.6.

2.5.5.6 Statistical analysis

All determinations were made in triplicate. To avoid changes resulting from different moisture contents or the addition of ingredients other than wheat flour or wholegrain rye flour, all the concentrations of ENNs and BEA were adjusted on the basis of respective flour used for bread making (i.e. µg/kg flour equivalent). The results were expressed as means ± SD. Data analysis was conducted by one-way analysis of variance (ANOVA) to assess whether significant ($p < 0.05$) variations existed using PASW Statistics 18.0 (SPSS Inc., Chicago, USA). Least significant difference (LSD) (equal variance assumed) or Tamhane (equal variance not assumed) post hoc test was performed when significant differences were found between the means.

2.2.6 Occurrence of ENNs and BEA in Chinese medicinal herbs

2.2.6.1 Sample extraction

All the samples were ground into fine particles using a laboratory mill and homogenized before extraction. Of each sample, 1 g was weighed and spiked with 10 ng (100 µL of a 100 ng/mL solution in ACN) of each of the labeled internal standards. After evaporation of the solvent, 10 mL of MeOH was added to each sample. The suspension was homogenized with a mixer (Hong-Hua, SK-1, Jiangsu, China) and extracted for 1.5 h on a shaker (160 rpm). Afterwards, the samples were centrifuged at 4800 rpm for 10 min, and filtered through a 0.22 µm membrane filter prior to HPLC. For samples that exceeded the upper linear range (10) of calibration curves, a second extraction was carried out, in which 1 g of the sample was spiked with 100 ng (100 µL of a 1 µg/mL solution in ACN) of each of the labeled internal standards, and the rest steps were the same as mentioned above.

2.2.6.2 Analysis of ENNs and BEA

Analysis of ENNs and BEA were carried out on a Shimadzu LC system interfaced to a mass spectrometer. A YMC-Pack ProC18 column was used for compound separation. The LC-MS/MS conditions were identical with those mentioned in section 2.2.4.6.

2.2.6.3 Method validation

Due to the variable and complex components of the Chinese medicinal herbs, a mixture of four blank samples was used for method validation. To be representative for all the analyzed samples, the blank surrogate mixture was made from lotus leaf (representative for leaf), chrysanthemum flower (representative for flower), kudzuvine root (representative for root), and black seasame (representative for seed), all of which were free of ENNs and BEA. For the determination of LODs, LOQs, as well as for recoveries, 1 g of the mixture was spiked with labeled ENNs and BEA at four different levels (2, 5, 15, and 20 µg/kg) in triplicate. LODs and LOQs were calculated by the method of Vogelgesang and Hädrich (1998). Intraday precision was determined with 5 measurements within the same day, and interday precision was determined with triplicate measurements in 3 different days within 2 weeks.

3. Results

3.1 Screening of ENNs and BEA-producing fungi strains

In search of potential producers of ENNs and BEA, a total of 57 fungal strains belonging to 4 different species of *Fusarium* genus (*F. proliferatum*, *F. oxysporum*, *F. sambucinum*, and *F. fujikuroi*) were screened on rice medium. Among the 57 strains, 7 were found to be relatively potent producers of ENNs and/or BEA.

In the subsequent step, an appropriate synthetic medium, all N-sources of which can be controlled was chosen. The rationale for this approach was to replace all the naturally abundant ^{14}N-sources in the culture medium by ^{15}N-sources in order to produce ^{15}N-labeled ENNs and BEA. For this purpose, Czapek-Dox liquid minimal medium, with $(NH_4)_2SO_4$ and $NaNO_3$ as its N-sources, was chosen to further cultivate the 7 relatively high-yielding strains screened on rice medium. The Czapek-Dox medium was then optimized with the most potent ENNs and BEA-producers.

3.1.1 Fungal culture on rice media and extraction of ENNs and BEA

To begin with, the effect of two types of rice media, as well as two sample preparation methods on ENNs and/or BEA was tested. For this purpose, 6 randomly selected *Fusarium* strains (*F. proliferatum* 4.0227, *F. proliferatum* 4.2098, *F. sambucinum* 4.0278, *F. sambucinum* 4.0304, *F. fujikuroi* 4.0628, and *F. oxysporum* 4.0473) were grown on rice media containing 60% or 100% of additional water, then extracted with ACN-H_2O (84:16, v/v). Subsequently, two sample preparation methods were tested: (a) alumina-charcoal-celite (5:7:3) cartridge cleanup according to Trucksess et al. (1987); (b) without cleanup, the so-called "dilute-and-shoot" approach.

As none of the 6 strains produced ENNs, only the levels of BEA are compared. The peak areas of BEA obtained from LC-MS/MS analysis are listed in **Table 3-1**. The effects of different water contents of rice medium on BEA production are shown in **Figure 3-1**, where the levels of BEA produced on rice medium with 100% of additional water are set as 1.0. The effects of the two sample preparation methods are compared in **Figure 3-2**, where the BEA contents after alumina-charcoal-celite (5:7:3) cartridge cleanup are set as 1.0.

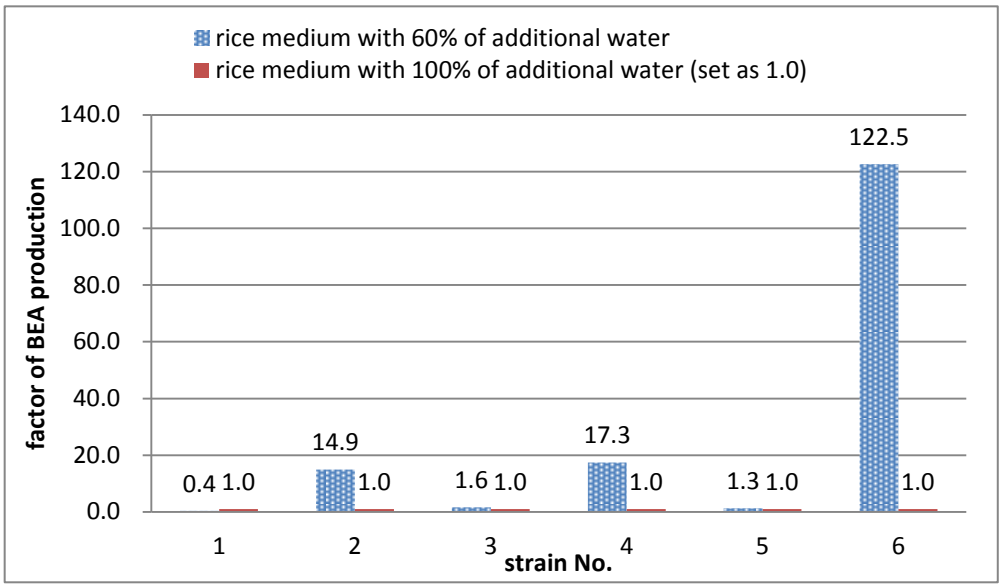


Figure 3-1. Comparison of BEA yields by 6 *Fusarium* strains cultivated on rice medium with 60% or 100% of additional water (sample preparation without cleanup)

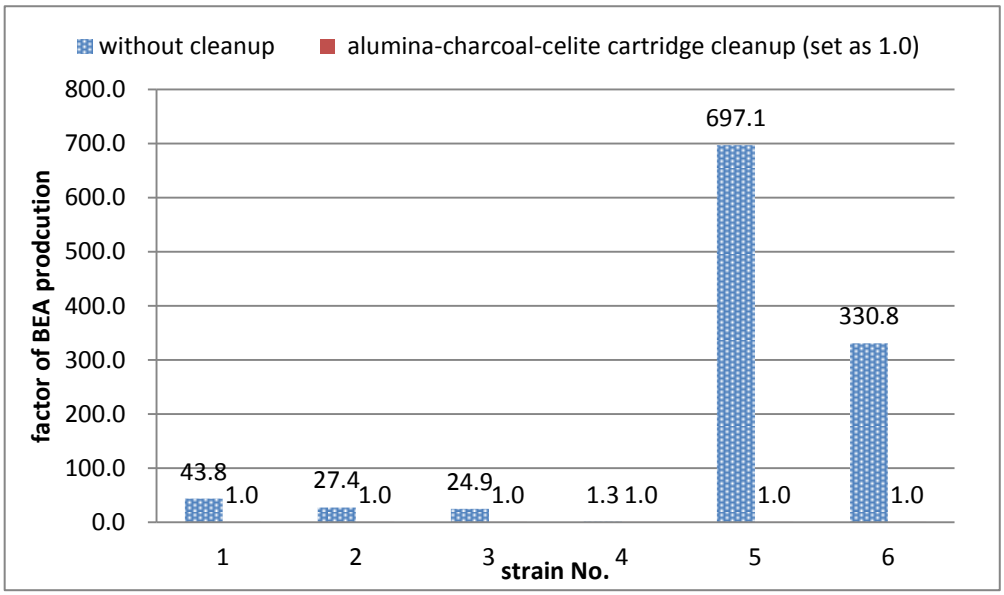


Figure 3-2. Comparison of BEA yields by 6 *Fusarium* strains after alumina-charcoal-celite (5:7:3) cartridge cleanup or without cleanup (cultivated on rice medium with 60% of additional water)

Table 3-1. Peak areas of BEA produced by 6 *Fusriuam* strains on rice media

No.	Strain	60% water rice dilute-and-shoot	100% water rice dilute-and-shoot	60% water rice alumina-charcoal-celite cartridge
1	*F. fujikuroi* 4.0628	2.86E+06	7.65E+06	6.53E+04
2	*F. oxysporum* 4.0473	1.92E+05	1.29E+04	7.00E+03
3	*F. proliferatum* 4.0227	4.01E+05	2.56E+05	1.61E+04
4	*F. proliferatum* 4.2098	1.56E+05	9.00E+03	1.16E+05
5	*F. sambucinum* 4.0278	4.28E+05	3.37E+05	6.14E+02
6	*F. sambucinum* 4.0304	4.73E+05	3.86E+03	1.43E+03

Figure 3-1 shows that 5 out of 6 *Fusarium* strains cultivated on rice medium with 60% of additional water gave better yields of BEA, while only strain No. 1 gave better yield of BEA when cultivated on rice medium with 100% of additional water. **Figure 3-2** shows that alumina-charcoal-celite (5:7:3) cartridge cleanup lead to major loss of BEA (in some cases, more than 95% loss).

Therefore, the rest of *Fusarium* strains listed in **Table 2-4** were cultivated on rice media containing 60% of additional water. For sample preparation, 2 g of the ground rice culture was extracted with 20 mL of ACN-H_2O (84:16, v/v), no further cleanup was applied to the crude extract except filtration through 0.22 μm membrane filter before HPLC analysis.

Among the total 57 strains screened, 7 strains including *F. oxysporum* 4.0629, *F.oxysporum* 4.1358, *F.oxysporum* 4.0226, *F. sambucinum* 4.0259, *F. sambucinum* 4.0979, *F. fujikuroi* 4.0628, and *F. fujikuroi* 4.0860 are found to be relatively potent producers of BEA and/or ENNs. The production of ENNs and BEA on rice medium (60% of additional water) by the 57 strains are shown in **Table 3-2**, with peak areas of ENNs and BEA obtained from LC-MS/MS analysis.

Table 3-2. Production of ENNs and BEA by 57 *Fusarium* strains on rice medium with 60% of additional water

Species	Strain No. (TMW)	ENN A	ENN A1	ENN B	ENN B1	BEA
F. proliferatum	4.0236	25	62	3940	112	1.24E+05
F. proliferatum	4.0227	34	34	261	11	4.01E+05
F. proliferatum	4.0827	136	186	819	25	15600
F. proliferatum	4.0828	12	87	18700	1240	96300
F. proliferatum	4.0840	37	50	384	50	1.16E+05
F. proliferatum	4.0948	25	62	738	25	56100
F. proliferatum	4.0954	298	285	360	62	25700
F. proliferatum	4.0984	25	12	1480	25	26000
F. proliferatum	4.1012	0	0	570	12	20700
F. proliferatum	4.1014	0	12	546	12	17100
F. proliferatum	4.1017	0	0	719	25	25600
F. proliferatum	4.1018	50	12	831	87	7480
F. proliferatum	4.2098	23	11	46	11	1.56E+05
F. proliferatum	4.2109	12	12	397	12	35900
F. oxysporum	4.0217	99	99	2820	248	90200
F. oxysporum	4.0473	46	0	205	0	1.92E+05
F. oxysporum	4.0629	47600	74700	5.60E+05	1.46E+05	1.51E+05
F. oxysporum	4.0136	3940	6560	45900	15700	9720
F. oxysporum	4.0116	6760	9380	23400	12100	2.23E+05
F. oxysporum	4.0148	74	161	6860	1220	0
F. oxysporum	4.1358	8.60E+06	8.26E+06	5.76E+06	5.68E+06	2.32E+05
F. oxysporum	4.0226	1.35E+07	2.17E+07	6.61E+07	3.53E+07	6.62E+05
F. oxysporum	4.0221	12900	25500	54100	34200	1.84E+05
F. oxysporum	4.1744	2950	5770	59000	9850	3.17E+05
F. oxysporum	4.2167	3670	4770	22500	10400	3570
F. oxysporum	4.0471	81	1440	7440	2620	1.33E+05
F. oxysporum	4.0470	0	12	3820	248	1.19E+05
F. oxysporum	4.0225	1820	2980	1.01E+05	9660	26400
F. oxysporum	4.0210	546	384	4920	267	1.30E+05

F. oxysporum	4.0228	360	248	1910	298	38400
F. oxysporum	4.0114	260	273	21100	1450	1.35E+05
F. oxysporum	4.0474	285	149	5920	893	1.16E+05
F. oxysporum	4.0472	62	25	2510	260	11100
F. oxysporum	4.1889	25	37	248	37	87600
F. oxysporum	4.0163	87	112	1560	236	3960
F. oxysporum	4.2169	62	50	1430	62	4120
F. oxysporum	4.2168	397	397	6520	1490	5360
F. oxysporum	4.2090	12	25	174	25	2120
F. sambucinum	4.0278	11	11	57	23	4.28E+05
F. sambucinum	4.0313	12	12	174	25	124
F. sambucinum	4.0181	37	99	4010	347	112
F. sambucinum	4.0259	0	3.47E+05	2.15E+07	1.90E+06	37
F. sambucinum	4.0304	11	11	34	11	4.73E+05
F. sambucinum	4.0264	25	707	53500	4770	1710
F. sambucinum	4.0321	0	149	41000	1600	15300
F. sambucinum	4.0165	217	26400	11400	0	3670
F. sambucinum	4.0796	25	50	5280	50	20300
F. sambucinum	4.0917	12	62	905	12	51300
F. sambucinum	4.0962	0	62	11100	12	8720
F. sambucinum	4.0976	74	13000	5200	161	7270
F. sambucinum	4.0977	25	1230	1.30E+05	4760	18000
F. sambucinum	4.0978	50	570	4220	260	36500
F. sambucinum	4.0979	335	2.05E+05	1.11E+05	92000	16100
F. fujikuroi	4.0336	74	1810	4440	1460	5900
F. fujikuroi	4.0628	23	34	352	11	2.86E+06
F. fujikuroi	4.0860	186	905	49200	7210	2.50E+06
F. fujikuroi	4.0953	124	236	3960	453	2.65E+05

3.1.2 Fungal culture in modified Czapek-Dox liquid minimal medium and extraction of ENNs and BEA

The 7 relatively potent ENNs/BEA-producing *Fusarium* strains screened from rice medium were further cultivated in the modified Czapek-Dox liquid minimal medium. Their yields of ENNs and BEA are given in **Table 3-3**. Although *F. oxysporum* 4.0226 produced the highest amounts of ENNs on rice medium, the most potent ENNs-producer found in the modified Czapek-Dox liquid minimal medium is another strain (*F. sambucinum* 4.0979). And the most potent BEA-producer in the modified Czapek-Dox liquid minimal medium is *F. fujikuroi* 4.0860, while *F. fujikuroi* 4.0628, which produced slightly higher amount of BEA on rice medium, yields the lowest BEA in the Czapek-Dox medium. Therefore, strains *F. sambucinum* 4.0979 and *F. fujikuroi* 4.0860 are selected as ENNs- and BEA-producers, respectively.

Table 3-3. Production of ENNs and BEA by 7 *Fusarium* strains in the Czapek-Dox medium

	ENN A	ENN A1	ENN B	ENN B1	BEA
F. oxysporum 4.0629	1.36E+03	2.67E+03	2.43E+04	5.06E+03	6.32E+02
F.oxysporum 4.1358	1.09E+04	1.20E+04	4.55E+04	1.90E+04	2.39E+04
F.oxysporum 4.0226	1.97E+03	1.79E+03	5.61E+03	2.01E+03	6.85E+03
F. sambucinum 4.0259	2.26E+04	1.43E+05	3.24E+06	5.23E+05	2.65E+03
F. sambucinum 4.0979	2.02E+07	1.71E+07	4.37E+07	1.61E+07	7.75E+03
F. fujikuroi 4.0628	1.30E+04	6.83E+03	5.27E+03	3.44E+03	1.24E+01
F. fujikuroi 4.0860	6.98E+04	4.17E+04	3.65E+04	2.25E+04	1.18E+08

As the type of nitrogen source as well as nitrogen concentration were reported to result in different ENNs yields (Audhya & Russell, 1974), the influence of $(NH_4)_2SO_4$ and $NaNO_3$ content on the production of BEA and ENNs was tested with *F. sambucinum* 4.0979 (ENNs-producer) and *F. fujikuroi* 4.0860 (BEA-producer). $(NH_4)_2SO_4$ was omitted in the medium and different amounts of $NaNO_3$ (1 g/L, 2 g/L, and 3 g/L) was added. Normal Czapek-Dox liquid minimal medium was used as control.

The amount of ENN A produced by *F. sambucinum* 4.0979 in different media is shown in **Figure 3-3**, and the amount of BEA produced by *F. fujikuroi* 4.0860 is shown in **Figure 3-4**, the values of control are set as 1.0, and the results are means of triplicate.

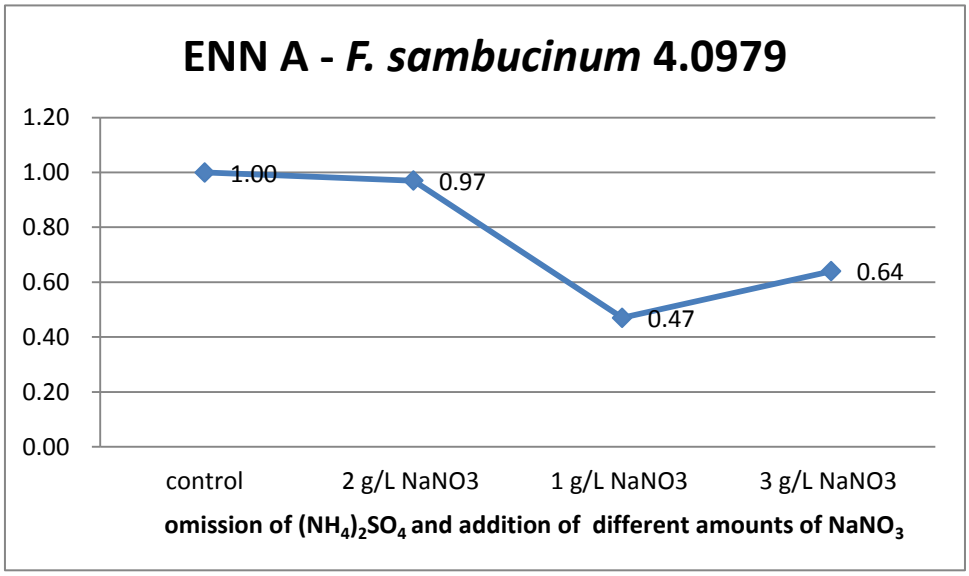


Figure 3-3. ENN A production by *F. sambucinum* 4.0979 in different Czapek-Dox media. The control medium contained trace of ammonium sulfate and 2 g/L sodium nitrate.

Figure 3-3 shows that for strain *F. sambucinum* 4.0979 (ENNs producer), the decrease of $NaNO_3$ (from 2 g/L to 1 g/L) in medium results in about 50% reduction of ENN A, and the increase of $NaNO_3$ (from 2 g/L to 3 g/L) also results in less ENN A, while the omission of $(NH_4)_2SO_4$ only leads to 3% decrease of ENN A production.

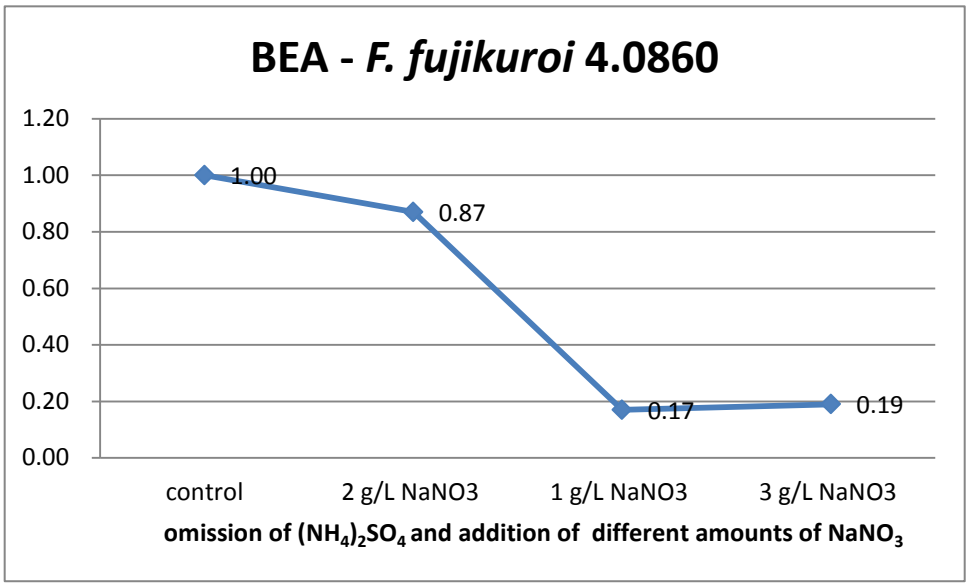


Figure 3-4. BEA production by *F. fujikuroi* 4.0860 in different Czapek-Dox media. The control medium contained trace of ammonium sulfate and 2 g/L sodium nitrate.

Figure 3-4 shows that for strain *F. fujikuroi* 4.0860 (BEA-producer), the omission of $(NH_4)_2SO_4$ leads to 13% decrease of BEA production, and that 2 g/L of $NaNO_3$ have the highest BEA yield compared to 1g/L or 3 g/L of $NaNO_3$.

As the omission of $(NH_4)_2SO_4$ did not have significant influence (only 3% or 13% decrease), in the subsequent biosynthesis of $^{15}N_3$-labeled ENNs and BEA, $(NH_4)_2SO_4$ was left out in the culture medium in order to avoid any ^{14}N-nitrogen source. And the original 2 g/L $NaNO_3$ concentration was kept as it gave highest yield of ENNs and BEA.

3.2 Biosynthesis of $^{15}N_3$-labeled ENNs and BEA

To produce ENNs, *F. sambucinum* strain 4.0979 was cultivated in the modified Czapek-Dox liquid minimal medium, of which the normal $Na^{14}NO_3$ was replaced by $Na^{15}NO_3$, and $(NH_4)_2SO_4$ was eliminated. The culture was incubated on a shaker at 25 ℃ for 7 d. A BEA-producer, *F. fujikuroi* strain 4.0860 was cultivated likewise to produce BEA.

The mycelia of the culture were extracted with ACN-H_2O (84:16, v/v). The crude extract was further processed by liquid-liquid extraction and solid phase extraction, and filtered through a 0.45 µm membrane filter prior to HPLC injection to isolate ENNs or BEA.

3.2.1 Preparation of $^{15}N_3$-labeled ENNs and BEA by HPLC

The procedure of the preparation of $^{15}N_3$-labeled ENNs by HPLC is presented here, and that of $^{15}N_3$-labeled BEA is not shown, since the two procedures are similar and the preparation of BEA is much simpler.

3.2.1.1 First round of HPLC for isolation of ENNs

HPLC method (a): mobile phase: MeOH-H_2O

The pre-processed extract of the fungal culture was injected into reversed-phase HPLC, and 4 major peaks were obtained with method (a).

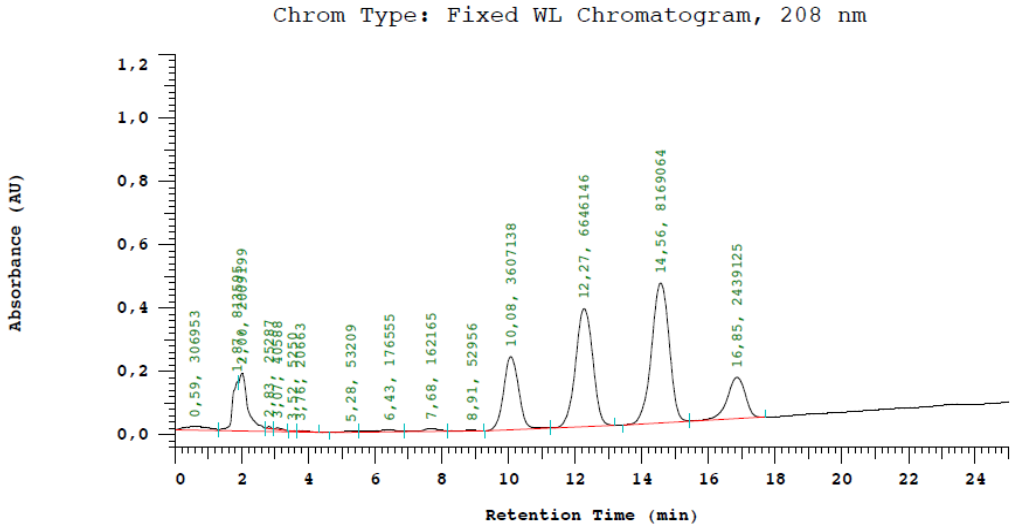


Figure 3-5. HPLC-DAD chromatogram of the extract by HPLC method (a)

Table 3-4. 4 major peaks obtained from HPLC method (a)

Peak No.	Rt (min)	Peak area
1	10.08	3.61E+06
2	12.27	6.65E+06
3	14.56	8.17E+06
4	16.85	2.44E+06

Each of the 4 major peaks were collected, and subjected to LC-MS/MS, and their composition of different ENNs (given in peak areas) is shown in **Table 3-5**.

Table 3-5. Composition of ENNs in the 4 major peaks collected from HPLC method (a)

Peak No.	ENN B	ENN B1	ENN A1	ENN A
1	8,07E+05	3.22E+05	4.79E+05	4.59E+05
2	2,15E+07	4.25E+06	1.16E+06	3.83E+05
3	1,22E+06	1.35E+07	6.22E+06	2.79E+06
4	5,50E+05	1.25E+06	1.58E+07	1.01E+07

The LC-MS/MS chromatograms of the 4 peaks are shown in **Figure 3-6**. The retention time of the ENNs during LC-MS/MS analysis are: ENN B (7.26 min), ENN B1 (7.97 min), ENN A1 (8.83 min), and ENN A (9.86 min).

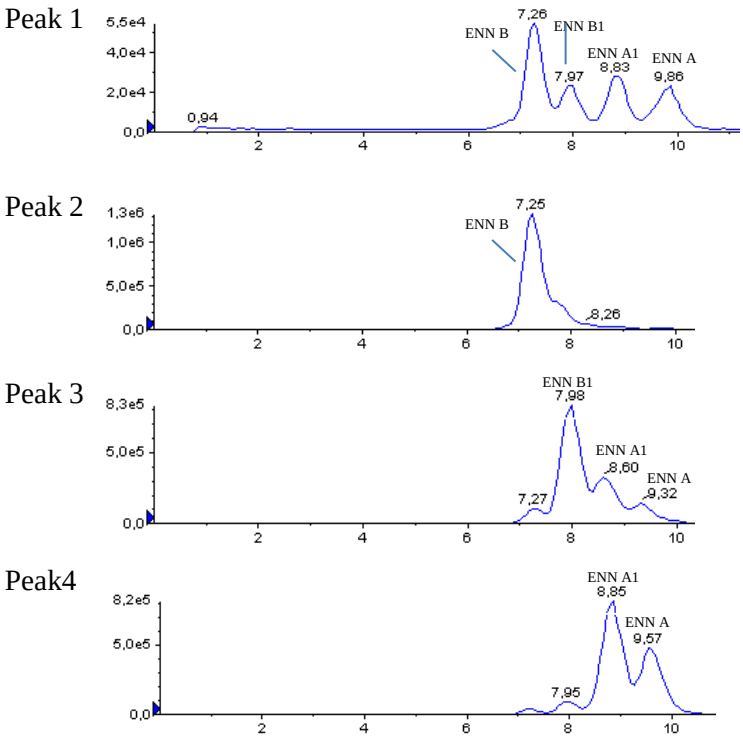


Figure 3-6. LC-MS/MS chromatograms of the 4 major peaks collected from HPLC method (a)

Table 3-5 and **Figure 3-6** show that the HPLC method (a) is not effective in separating the different ENNs, as only ENN B is predominating in one particular peak (Peak 2), while none of ENNs A, A1, and B1 is predominating in one certain peak. Therefore, method (a) is not suitable for isolating different ENNs from the extract.

HPLC method (b): mobile phase: ACN-H_2O

The extract was injected into HPLC, and 9 major peaks were obtained with method (b).

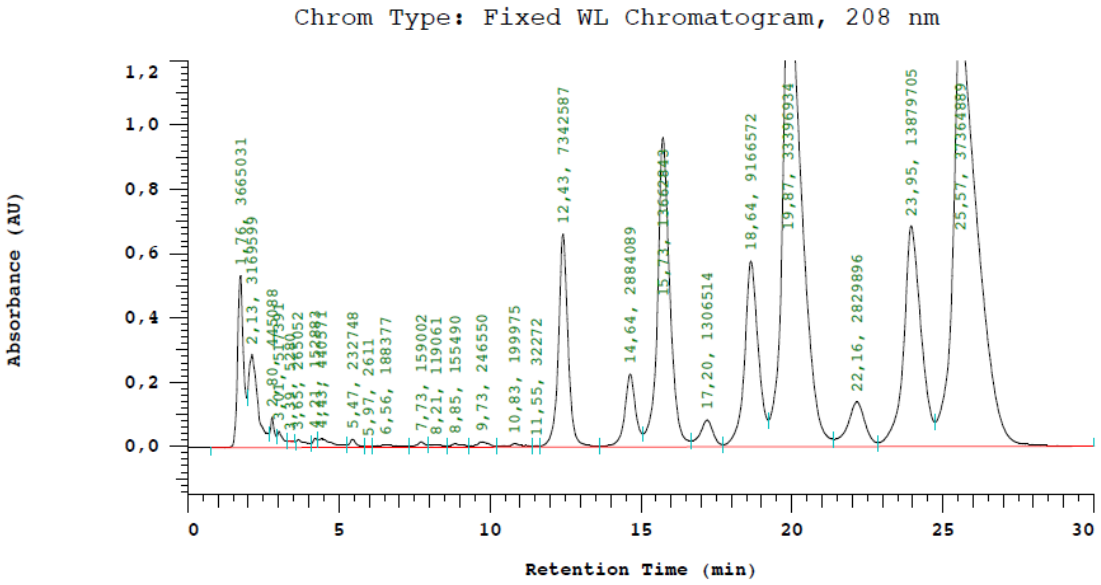


Figure 3-7. HPLC-DAD chromatogram of the extract by HPLC method (b)

Table 3-6. 9 major peaks obtained from HPLC method (b)

Peak No.	Rt (min)	Peak area
1	12.4	7.34E+06
2	14.6	2.88E+06
3	15.7	13.66E+06
4	17.2	1.31E+06
5	18.6	9.17E+06
6	19.9	33.39E+06
7	22.2	2.83E+06
8	23.95	13.88E+06
9	25.6	37.36E+06

Each of the 9 major peaks were collected, and measured by LC-MS/MS to check their composition of the ENNs. The LC-MS/MS analysis reveals that peak 1, 3, 6, and 9 show similar fragmentation pattern and retention time with that of ENN B, ENN B1, ENN A1, and ENN A standards, respectively. The composition of ENNs in peaks 1, 3, 6, and 9 (given in peak areas) are shown in **Table 3-7**. The other peaks are not shown here as they either contain no or only traces of ENNs.

Table 3-7. Composition of ENNs in peaks 1, 3, 6, and 9 collected from HPLC method (b)

Peak No.	ENN B	ENN B1	ENN A1	ENN A
1	1,10E+07	2,18E+06	2,79E+05	2,32E+05
3	1,05E+06	2,68E+07	6,03E+05	2,71E+05
6	6,12E+05	2,65E+06	8,91E+06	2,80E+05
9	2,54E+05	2,33E+05	1,09E+06	1,77E+07

The LC-MS/MS chromatograms of the peaks 1, 3, 6, and 9 are shown in **Figure 3-8**. The retention times of the ENNs during LC-MS/MS analysis are: ENN B (7.23 min), ENN B1 (7.91 min), ENN A1 (8.56 min), and ENN A (9.63 min).

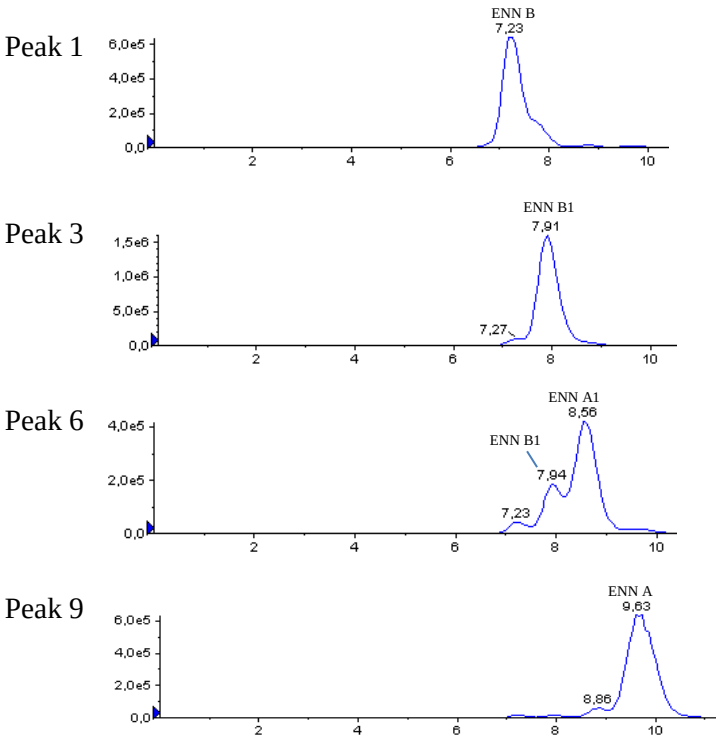


Figure 3-8. LC-MS/MS chromatograms of Peaks 1, 3, 6, and 9 collected from HPLC method (b)

3.2.1.2 Second round of HPLC for isolation of ENNs

The peaks 1, 3, 6, and 9 collected from the 1st round of HPLC using method (b) were further purified with a 2nd round of HPLC. The chromatograms of each peak are shown in **Figures 3-9 to 3-12**, the major peak (retention time: 8–14 min) in each chromatogram was collected.

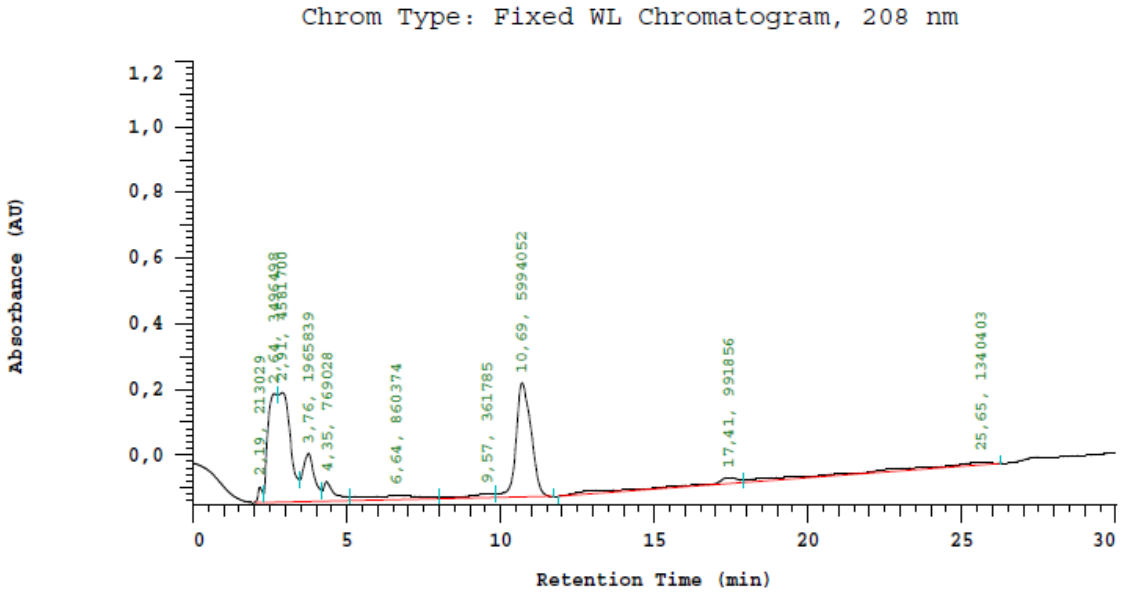


Figure 3-9. 2nd purification of Peak 1

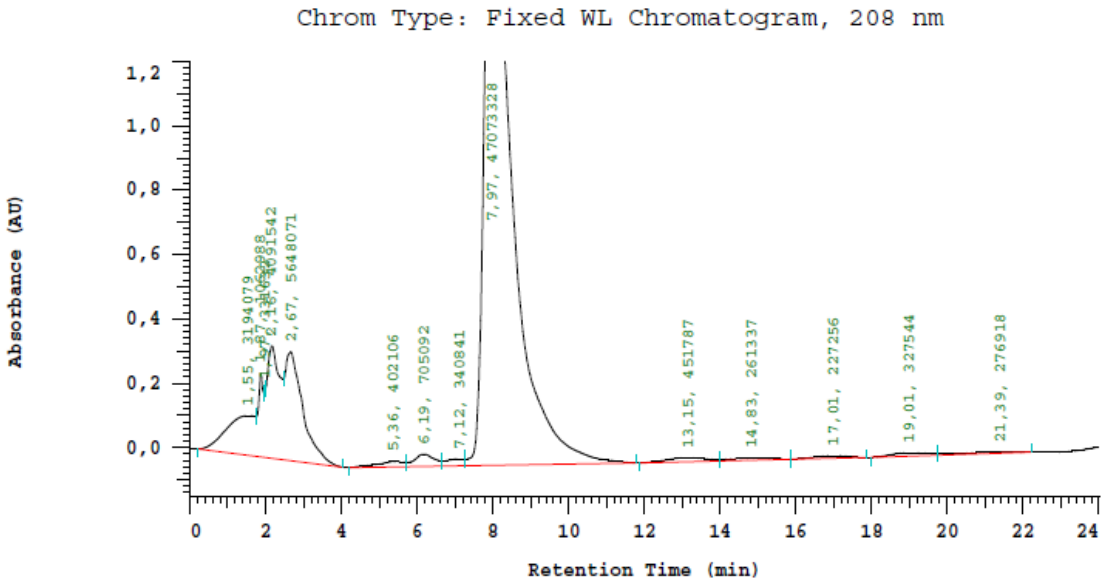


Figure 3-10. 2nd purification of Peak 3

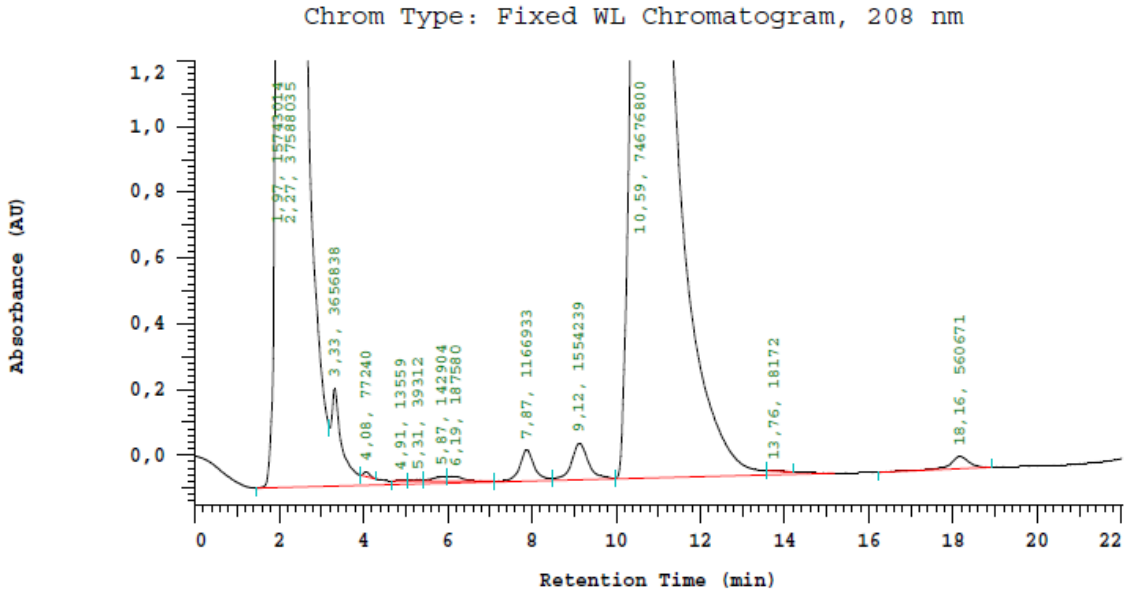


Figure 3-11. 2nd purification of Peak 6

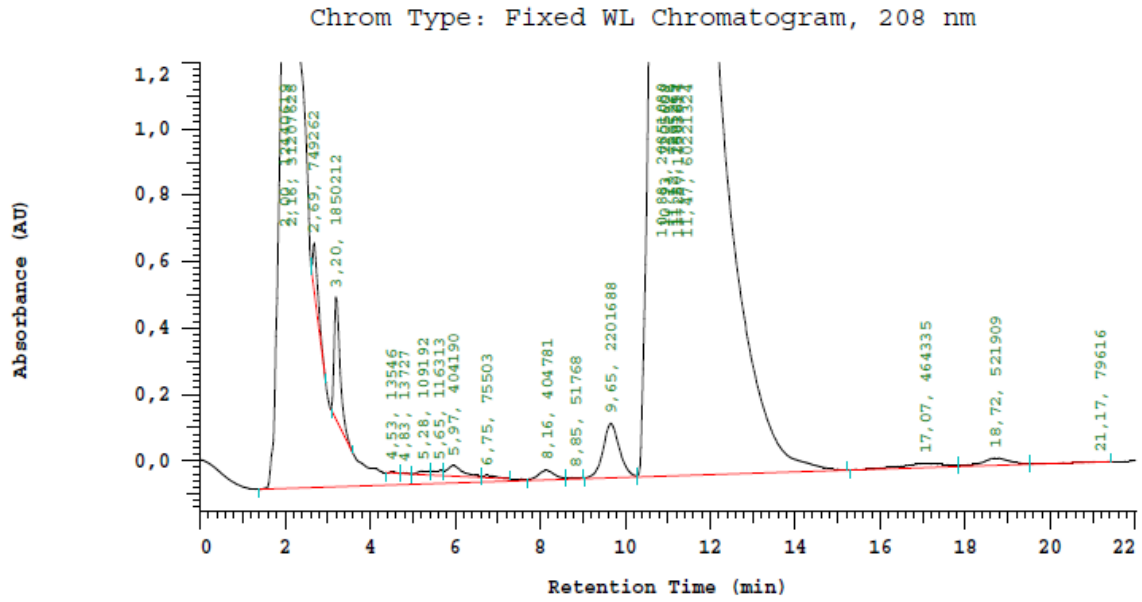


Figure 3-12. 2nd purification of Peak 9

3.2.1.3 LC-MS analysis of the rechromatographed peaks

For ENNs, each of the rechromatographed 4 peaks was analyzed by LC-MS in the full scan mode to check its purity. The LC-MS full scan chromatogram of the rechromatographed Peak 3 is shown in **Figure 3-13 (A & B)** as a representative. **Figure 3-13 (A)** presents the MS between 7.857–7.890 min, with signal of *m/z* 657.8 corresponding to ENN B1. **Figure 3-13 (B)** gives the extracted MS chromatogram between *m/z* 600–900.

As for BEA, only one major peak was collected from the 1st round of HPLC preparation, and this peak was further purified by a 2nd round of HPLC, the rechromatographed peak (referred to as Peak 1-"BEA") was also analyzed by LC-MS in the full scan mode to cheack its purity.

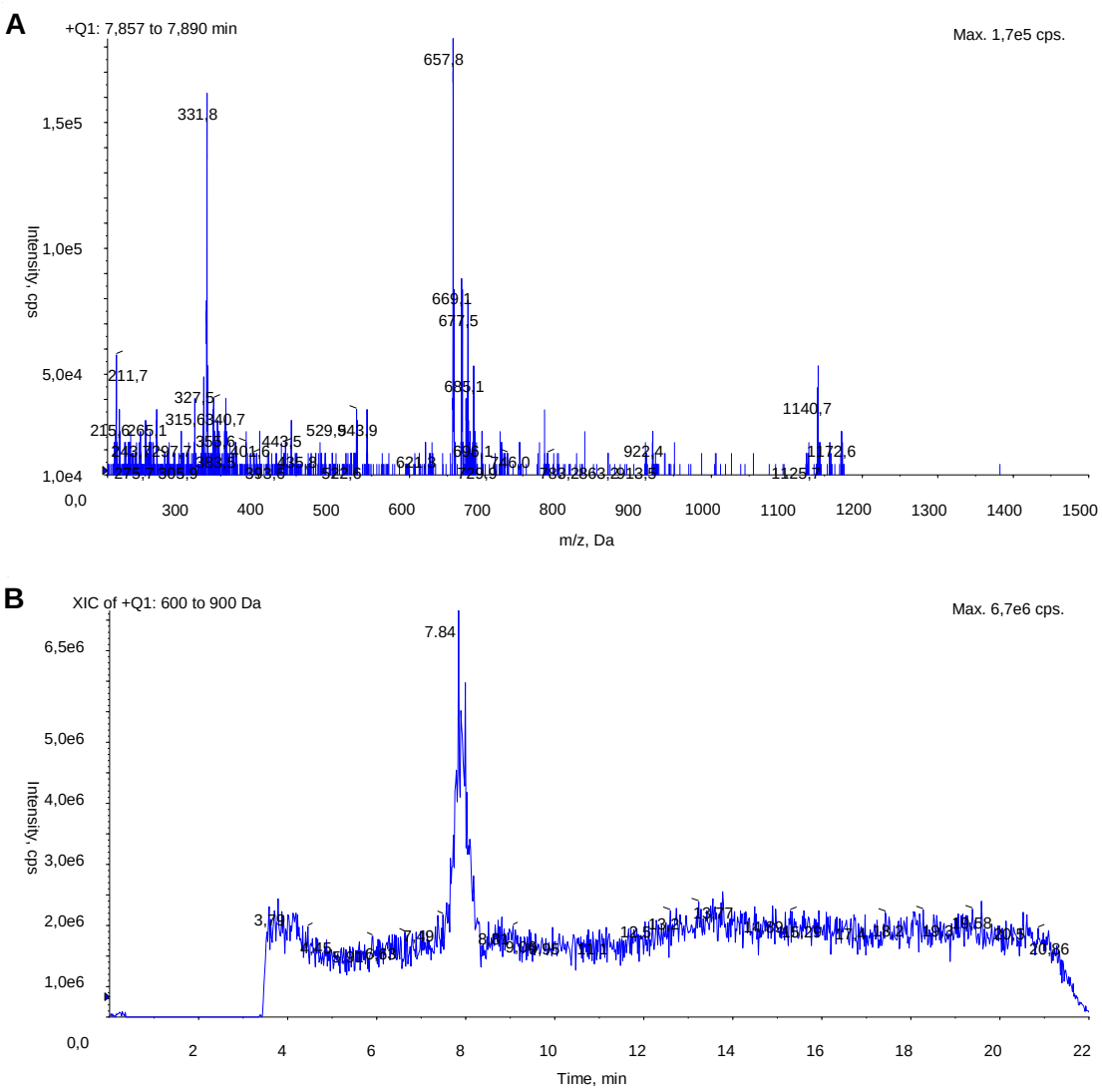


Figure 3-13. LC-MS full scan of the rechromatographed Peak 3: **(A)** MS signals between 7.857–7.890 min, with signal of *m/z* 657.8 corresponding to ENN B1; **(B)** Extracted MS chromatogram between *m/z* 600–900

3.2.2 ^{1}H-NMR of $^{15}N_3$-labeled ENNs and BEA

The ^{1}H-NMR chemical shifts for ENNs and BEA, given in δ/ppm (TMS) are listed in **Table 3-8** to **Table 3-12**. The data is in good agreement with that from literature.

Table 3-8. ^{1}H-NMR chemical shift assignments for Peak 9 (ENN A)

		ENN A (Blais et al., 1992)	**Peak 9**
N-Me-Ile[a]	αH	4.65 (d, 3H)	4.68 (d, 3H) (*J* = 6.8)
	βH	2.04 (m, 3H)	2.09 (m, 3H)
	$\gamma_1(CH_2)$	1.04 (3H)	1.03 (bs, 3H)
		1.42 (3H)	1.44 (m, 3H)
	$\gamma_2(CH_3)$	1.00 (9H)	1.02 (d, *J* = 3.8, 9H)
	$\delta(CH_3)$	0.84 (9H)	0.89 (m, 9H)
	N-CH_3	3.09 (s, 9H)	3.13 (s, 9H)
Hiv[b]	αH	5.10 (d, 3H)	5.14 (d, *J* = 8.1, 3H)
	βH	2.25 (3H)	2.28 (m, 3H)
	$\gamma(CH_3)$	0.92 (9H)	0.91–1.01 (m, 18H)
		0.98 (9H)	

[a]**N-Me-Ile**: *N*-methylisoleucine; [b]**Hiv**: D-2-hydroxyisovaleric acid

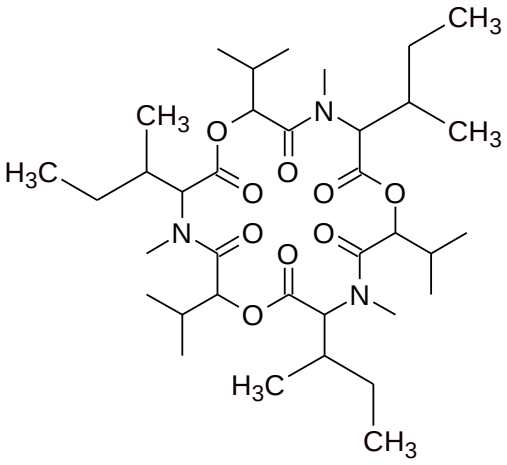


Figure 3-14. Chemical structure of ENN A

Table 3-9. ^{1}H-NMR chemical shift assignments for Peak 6 (ENN A1)

		ENN A1		
		(Blais et al., 1992)	(Xu, 1993)	**Peak 6**
N-Me-Ile[a]	αH	4.62	4.71 (d, 1H)	4.70 (m, 2H)
		4.66	4.69 (d, 1H)	
	βH	2.01	2.06 (t, 2H)	2.03 (m, 2H)
	$\gamma_1(CH_2)$	1.04	1.08 (m, 2H)	1.04 (s, 2H)
		1.42	1.41 (m, 2H)	1.42 (t, *J* = 7.3, 2H)
	$\gamma_2(CH_3)$	0.98	1.00 (d, 6H)	1.03 (d, *J* = 6.0, 6H)
	$\delta(CH_3)$	0.83	0.86 (t, 6H)	0.90 (m, 6H)
	N-CH_3	3.08	3.11 (s, 6H)	3.17 (s, 6H)
N-Me-Val[b]	αH	4.42	4.45 (d, 1H)	4.52 (d, *J* = 9.8, 1H)
	βH	2.28	2.31 (m, 1H)	2.22 (m, 1H)
	$\gamma(CH_3)$	1.03	1.07 (d, 3H)	1.09 (d, *J* = 6.6, 3H)
		0.87	0.90 (d, 3H)	0.92 (s, 3H)
	N-CH_3	3.10	3.14 (s, 3H)	3.19 (s, 3H)
Hiv[c]	αH	5.10	5.15; 5.12; 5.09 (d, 3H)	5.09 (m, 3H)
	βH	2.26	2.26 (m, 3H)	2.22 (m, 3H)
	$\gamma(CH_3)$	0.92–0.99	0.94–1.00 (18H)	0.96–1.02 (m, 18H)

[a]**N-Me-Ile**: *N*-methylisoleucine; [b]**N-Me-Val**: *N*-methylvaline; [c]**Hiv**: D-2-hydroxyisovaleric acid

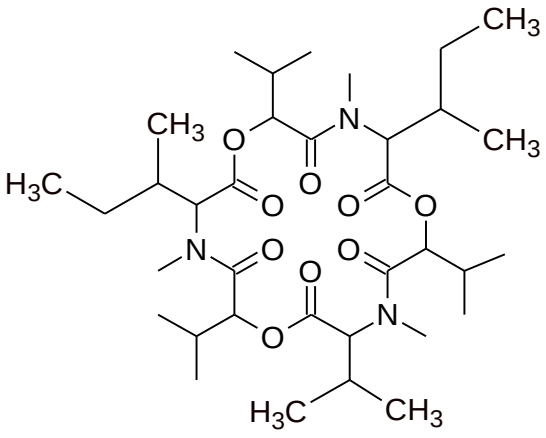


Figure 3-15. Chemical structure of ENN A1

Table 3-10. ^{1}H-NMR chemical shift assignments for Peak 1 (ENN B)

		ENN B (Xu, 1993)	**Peak 1**
N-Me-Val[a]	αH	4.51 (d, 3H)	4.53 (d, *J* = 12.0, 3H)
	βH	2.29 (m, 3H)	2.29 (m, 3H)
	γ(CH_3)	0.88 (d, 9H)	0.93 (d, *J* = 6.6, 9H)
		1.05 (d, 9H)	1.09 (d, *J* = 6.6, 9H)
	N-CH_3	3.12 (s, 9H)	3.17 (s, 9H)
Hiv[b]	αH	5.13 (d, 3H)	5.13 (d, *J* = 6.2, 3H)
	βH	2.26 (m, 3H)	2.26 (m, 3H)
	γ(CH_3)	0.95 (d, 9H)	0.97 (d, *J* = 6.8, 9H)
		0.97 (d, 9H)	1.01 (d, *J* = 6.6, 9H)

[a]**N-Me-Val**: *N*-methylvaline; [b]**Hiv**: D-2-hydroxyisovaleric acid

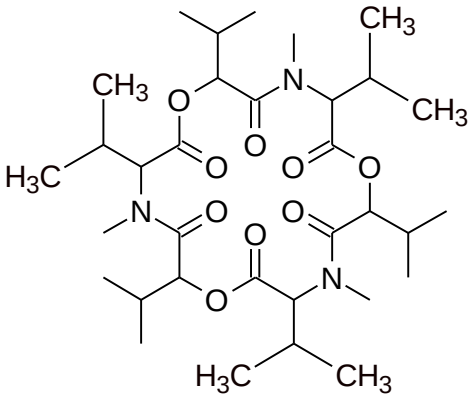


Figure 3-16. Chemical structure of ENN B

Table 3-11. ^{1}H-NMR chemical shift assignments for Peak 3 (ENN B1)

		ENN B1		
		(Blais et al., 1992)	(Xu, 1993)	**Peak 3**
N-Me-Val[a]	αH	4.42	4.50 (d, 1H)	4.51 (d, *J* = 9.2, 1H)
		4.46	4.45 (d, 1H)	4.47 (d, *J* = 8.1, 1H)
	βH	2.27	2.30 (m, 2H)	2.30 (m, 2H)
	γ(CH_3)	1.02	1.05 (6H)	1.08 (d, *J* = 6.6, 6H)
		0.87	0.88 (6H)	0.89 (m, 6H)
	N-CH_3	3.10	3.12 (s, 6H)	3.14 (s, 6H)
N-Me-Ile[b]	αH	4.65	4.72 (d, 1H)	4.73 (d, *J* = 12.8, 1H)
	βH	2.04	2.07 (m, 1H)	2.07 (m, 1H)
	γ_1(CH_2)	1.04	1.10 (m, 1H)	1.03 (m, 1H)
		1.42	1.41 (m, 1H)	1.43(m, 1H)
	γ_2(CH_3)	0.98	0.99 (3H)	1.01 (m, 3H)
	δ(CH_3)	0.82	0.86 (3H)	0.87 (m, 3H)
	N-CH_3	3.08	3.10 (s, 3H)	3.12 (s, 3H)
Hiv[c]	αH	5.10	5.15; 5.12; 5.09 (3H)	5.16 (m, 3H)
	βH	2.26	2.27 (d, 3H)	2.30 (m, 3H)
	γ(CH_3)	0.92–0.99	0.93–0.99 (18H)	0.90–1.01 (m, 18H)

[a]**N-Me-Val**: *N*-methylvaline; [b]**N-Me-Ile**: *N*-methylisoleucine; [c]**Hiv**: D-2-hydroxyisovaleric acid

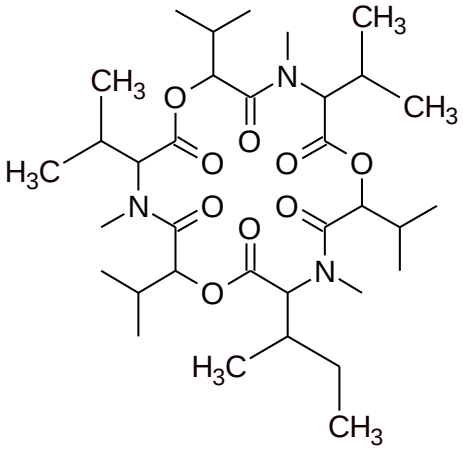


Figure 3-17. Chemical structure of ENN B1

Table 3-12. ^{1}H-NMR chemical shift assignments for Peak 1-"BEA" (BEA)

		BEA		
		Gupta et al., 1991	Hamill et al., 1969	Peak 1-"BEA"
N-Me-Phe[a]	αH	5.42 (dd, 3H)	5.60 (q, X-proton)	5.47 (m, 3H)
	βH	2.96 (m, 3H, β-C<u>H</u>(H))	3.02 (one of AB proton)	2.89 (m, 3H)
		3.34 (dd, 3H, β-CH(<u>H</u>))	3.39 (one of AB proton)	3.32 (m, 3H)
	aromatic H	7.23 (m, 3*5H)	7.25 (s,3*5H)	7.16 (m, 15H)
	N-CH_3	2.98 (s, 9H)	3.04 (s, 9H)	2.95 (s, 9H)
Hiv[b]	αH	4.93 (d, 3H)	4.87 (d, 3H)	4.80 (d, *J* = 8.1, 3H)
	βH	2.03 (m, 3H)	1.95 (m, 3H)	1.89 (m, 3H)
	γ(CH_3)	0.42 (d, 9H)	0.4 (d, 9H)	0.34 (d, *J* = 6.8, 9H)
		0.79 (d, 9H)	0.8 (d, 9H)	0.73 (d, *J* = 6.6, 9H)

[a]**N-Me-Phe**: *N*-methylphenylalanine; [b]**Hiv**: D-2-hydroxyisovaleric acid

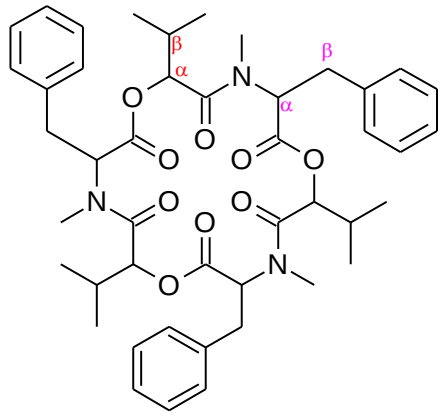


Figure 3-18. Chemical structure of BEA

3.2.3 Quantitation of $^{15}N_3$-labeled ENNs and BEA by qNMR

Determining the concentration of standard solutions of labeled ENNs and BEA is inaccurate by gravimetry due to the small amount of the mycotoxins isolated from fungal culture. The precise amount of commercially bought unlabeled ENNs and BEA was also unknown as the purity is not certified. Thus, quantitation by qNMR was adopted, using a caffeine sample of known concentration as external standard. The proton signals at 7.87 ppm (caffeine), 5.47 ppm ($[^{15}N]_3$-BEA), 5.14 ppm ($[^{15}N]_3$-ENN A), and 5.09 ppm ($[^{15}N]_3$-ENN A1) were chosen for quantitation (Appendix: qNMR spectra of $[^{15}N]_3$-ENN A, $[^{15}N]_3$-ENN A1, and $[^{15}N]_3$-BEA). By comparing the intensities of the latter three signals to that of the caffeine signal, the molar concentrations of the three mycotoxins are determined as follows: 1.0355 mmol/L ($[^{15}N]_3$-ENN A), 1.1203 mmol/L ($[^{15}N]_3$-ENN A1), and 3.1019 mmol/L ($[^{15}N]_3$-BEA). As already reported for the ochratoxin A (Korn et al., 2011), qNMR proved again to be a suitable and accurate tool in mycotoxin quantitation.

Based on the molar concentrations of the $^{15}N_3$-labeled ENN A, ENN A1, and BEA, the total yields of the microbiological production are calculated to be 430 µg ($[^{15}N]_3$-ENN A), 450 µg ($[^{15}N]_3$-ENN A1), and 1460 µg ($[^{15}N]_3$-BEA). The exact total amounts of $[^{15}N]_3$-ENN B and $[^{15}N]_3$-ENN B1 were not quantitated by qNMR, instead, these two ENNs were prepared into standard solutions, and the concentrations of the solutions were determined by comparing their UV absorption with that of $^{15}N_3$-labeled ENN A and ENN A1. The total amounts of $[^{15}N]_3$-ENN B and $[^{15}N]_3$-ENN B1 are roughly estimated to be 100 and 210 µg, respectively.

3.2.4 Analysis of unlabeled and labeled ENNs and BEA by LC-MS/MS

Detection of the analytes was carried out by ESI-(+)-MS/MS, product ion scans of ENNs and BEA standards were recorded using the protonated molecules as parent ions. Generally, the labeled standards give similar fragmentation patterns to the respective unlabeled compounds. As displayed in **Figure 3-19**, the three most intense fragments derive from $[M+H]^+$ ion (*m/z* 668) of unlabeled ENN A1 are *m/z* 196, *m/z* 210, and *m/z* 228, similar fragments are produced by $[M+H]^+$ ions (*m/z* 671) of labeled ENN A1, with *m/z* 197, *m/z* 211, and *m/z* 229 being the three most intense signals. The fragmentation of labeled and unlabeled BEA is shown in **Figure 3-20**. In comparison to the protonated

molecules that contain a mass increment of three being in accordance with the three [^{15}N] incorporated, the fragments contains only a mass increment of one equivalent to one [^{15}N] incorporated.

Based on this information, a fragmentation pathway of ENNs and BEA is proposed, with the protonated molecule in the center of **Figure 3-21** showing an imaginary molecule composed of all side chains incorporated in the different ENNs and BEA. In accordance with the observed occurrence of one labeled nitrogen in each fragment, the fragments obviously contain one amino acid moiety. Hypothetically, the ring of the molecule had an even chance to break in either of the three marked C-O bonds. Due to the different substituents on the amino acid residues, ENNs and BEA result in different fragments. For BEA, the most plausible fragments are those containing phenylalanine residues (*m/z* 262), which then lose H_2O to give *m/z* 244. For ENN A, fragments of *m/z* 228 containing *sec*-butyl moieties are formed after break of the ring, and subsequent loss of H_2O results in *m/z* 210. Similarly, fragments of *m/z* 214 and *m/z* 196 are obtained from ENN B. For ENNs A1 and B1 which contain both isopropyl and *sec*-butyl side chains, a mixture of *m/z* 196, *m/z* 214, *m/z* 210, and *m/z* 228 fragments is observed.

In previous studies, MS/MS fragmentations of ENNs and BEA were reported but no detailed explanations were given. In their LC-MS/MS method, Sørensen et al. (2008) used the fragments at *m/z* 555 and 210, at *m/z* 541 and 210, at *m/z* 527 and 196, at *m/z* 228 and 196, and at *m/z* 362 and 244 for ENN A, ENN A1, ENN B, ENN B1 and BEA, respectively. The masses of the quantifier ions (*m/z* 210, 196, 244) were assigned to protonated "monomers" with phenylmethyl, *sec*-butyl or isopropyl residues after loss of a water molecule, but no detailed structural suggestion for the fragments was given. Jestoi et al. (2009) also used the same fragments, with no information on their structure or on the route of formation. In the report by Sewram et al. (1999), the fragments of BEA were suggested to result from the cleavage of the amide bond, which could not be substantiated by the present study.

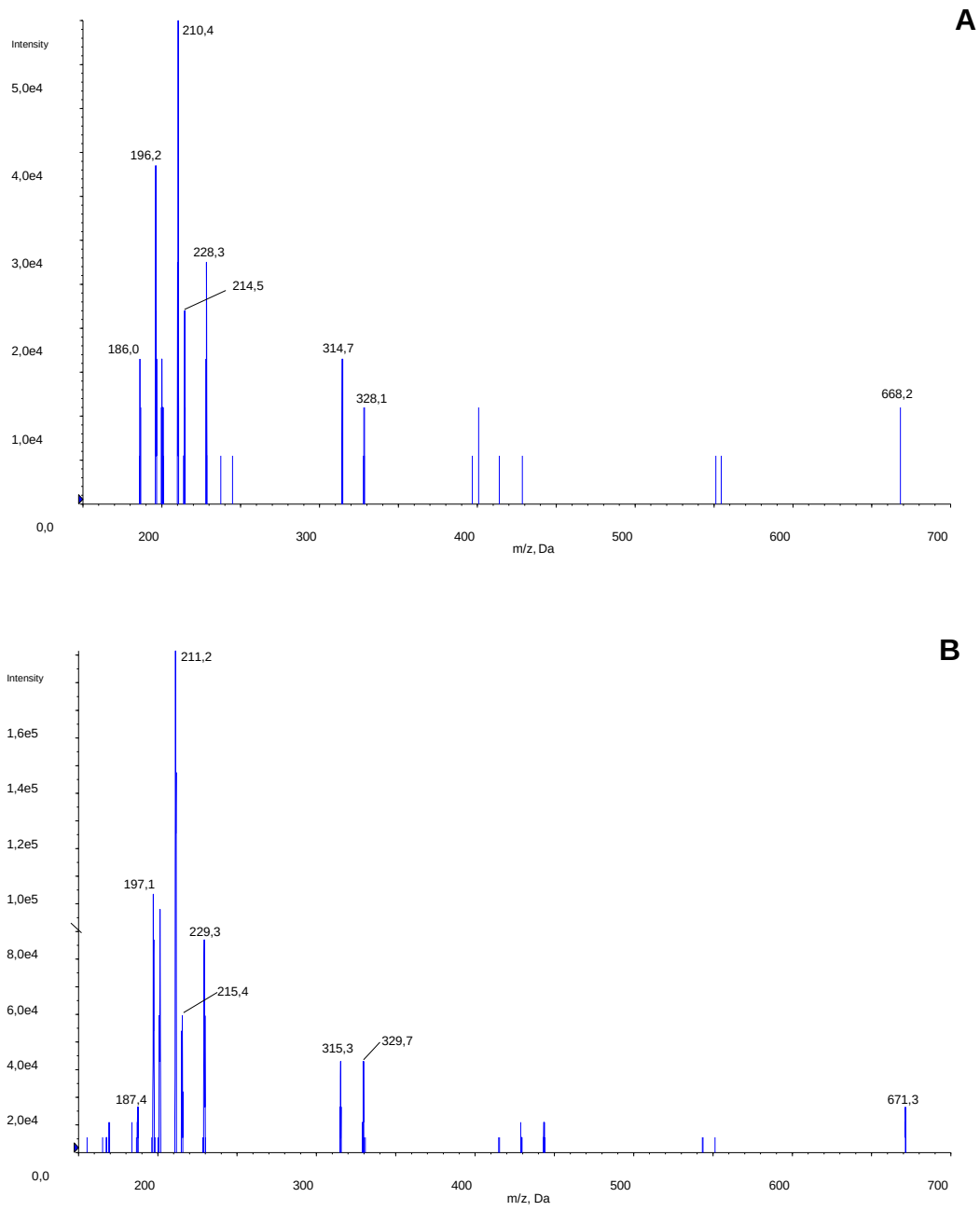


Figure 3-19. (**A**) ESI-(+)-LC-MS/MS spectrum of ENN A1 (precursor *m/z* = 668, $[M+H]^+$); (**B**) ESI-(+)-LC-MS/MS spectrum of $[^{15}N]_3$-labeled ENN A1 (precursor *m/z* = 671, $[M+H]^+$)

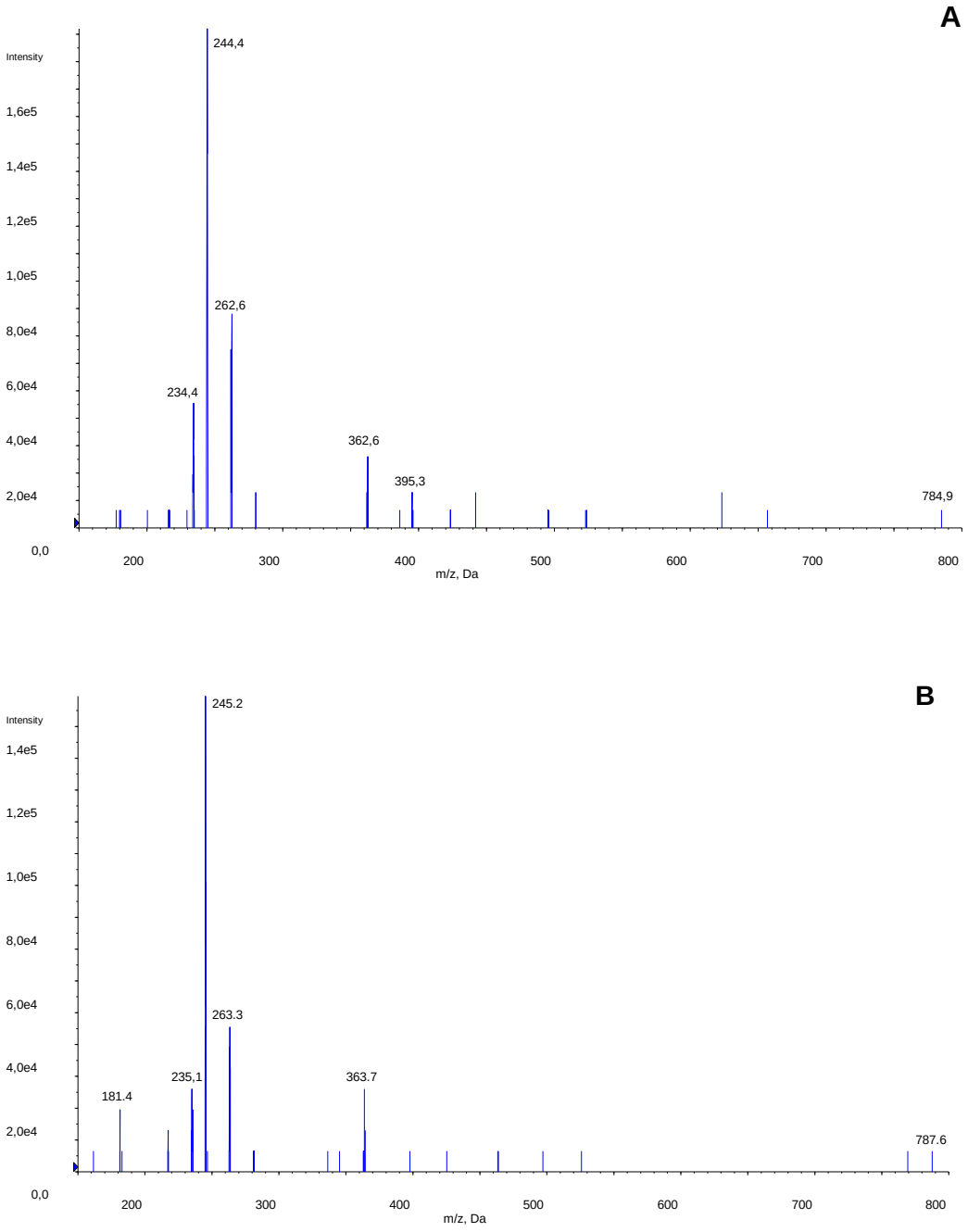


Figure 3-20. (**A**) ESI-(+)-LC-MS/MS spectrum of BEA (precursor *m/z* = 784, $[M+H]^+$); (**B**) ESI-(+)-LC-MS/MS spectrum of $[^{15}N]_3$-labeled BEA (precursor *m/z* = 787, $[M+H]^+$)

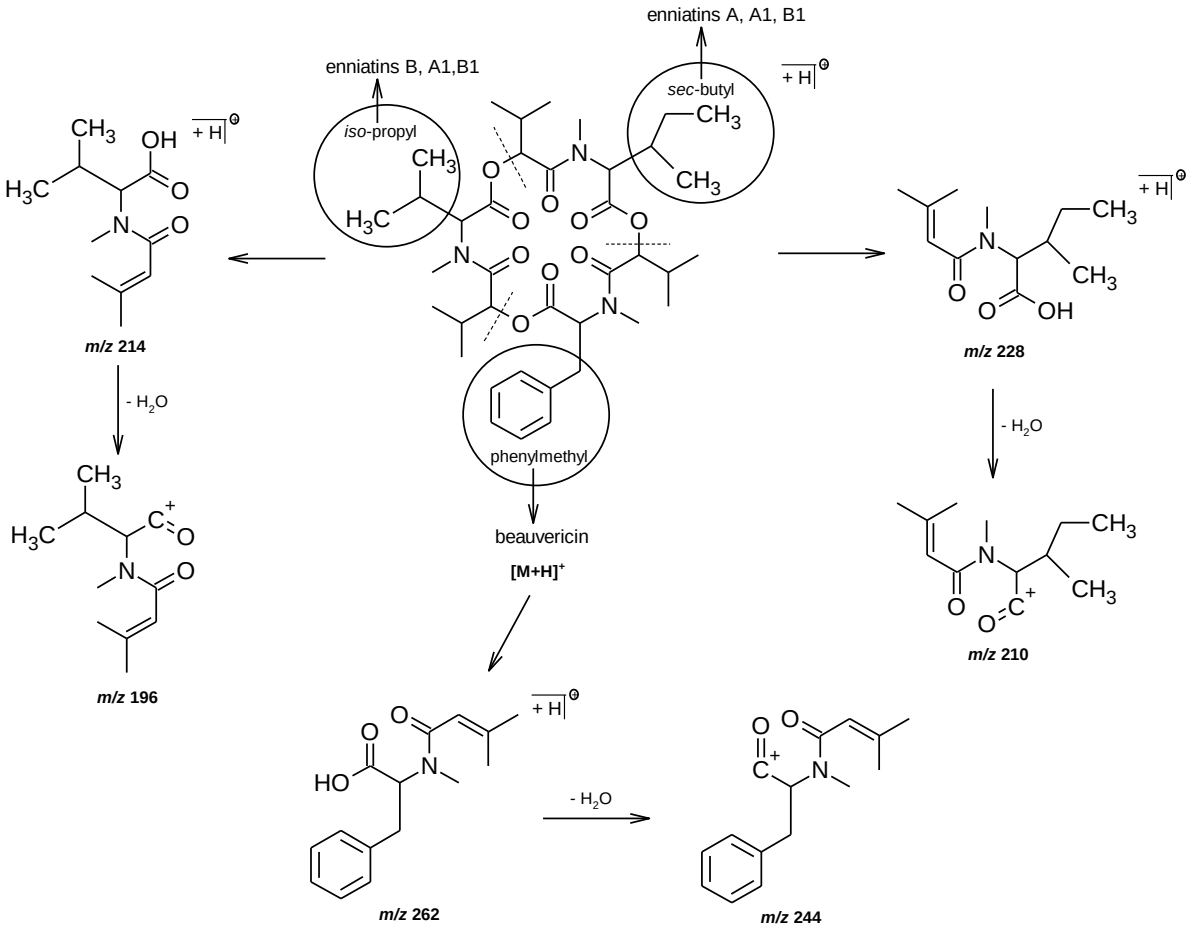


Figure 3-21. Proposed MS/MS fragmentation routes of ENNs and BEA. The depicted structure refers to a hypothetical molecule composed of the amino acids included in ENNs and BEA.

3.3 SIDAs of ENNs and BEA in cereals and related food

3.3.1 Sample preparation

A wheat sample (W1) was used to compare the performance of two sample preparation methods: (a) direct injection without SPE cleanup; (b) MycoSep 225 SPE cartridge cleanup. The HPLC-DAD chromatograms of the two samples are presented in **Figure 3-22**, which suggests that MycoSep 225 is not effective in removing the impurities being eluted in the retention range before 4 min. The HPLC-MS/MS chromatograms of the two samples are given in **Figure 3-23**, showing that the intensities of ENNs and BEA are lower after MycoSep 225 cleanup. Based on these results, no SPE cleanup was used in further experiments.

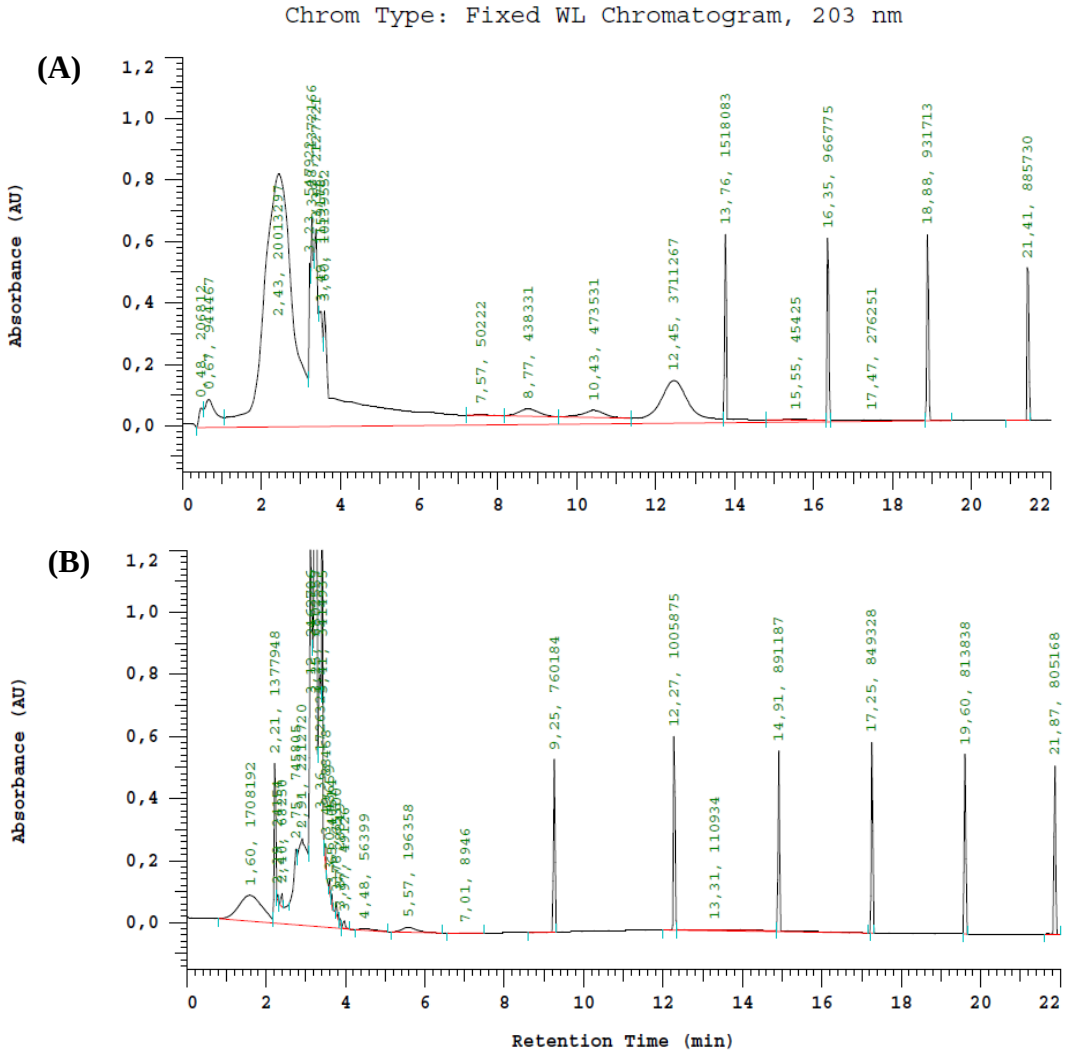


Figure 3-22. HPLC-DAD chromatograms of W1: **(A)** no SPE cleanup; **(B)** MycoSep 225 cleanup

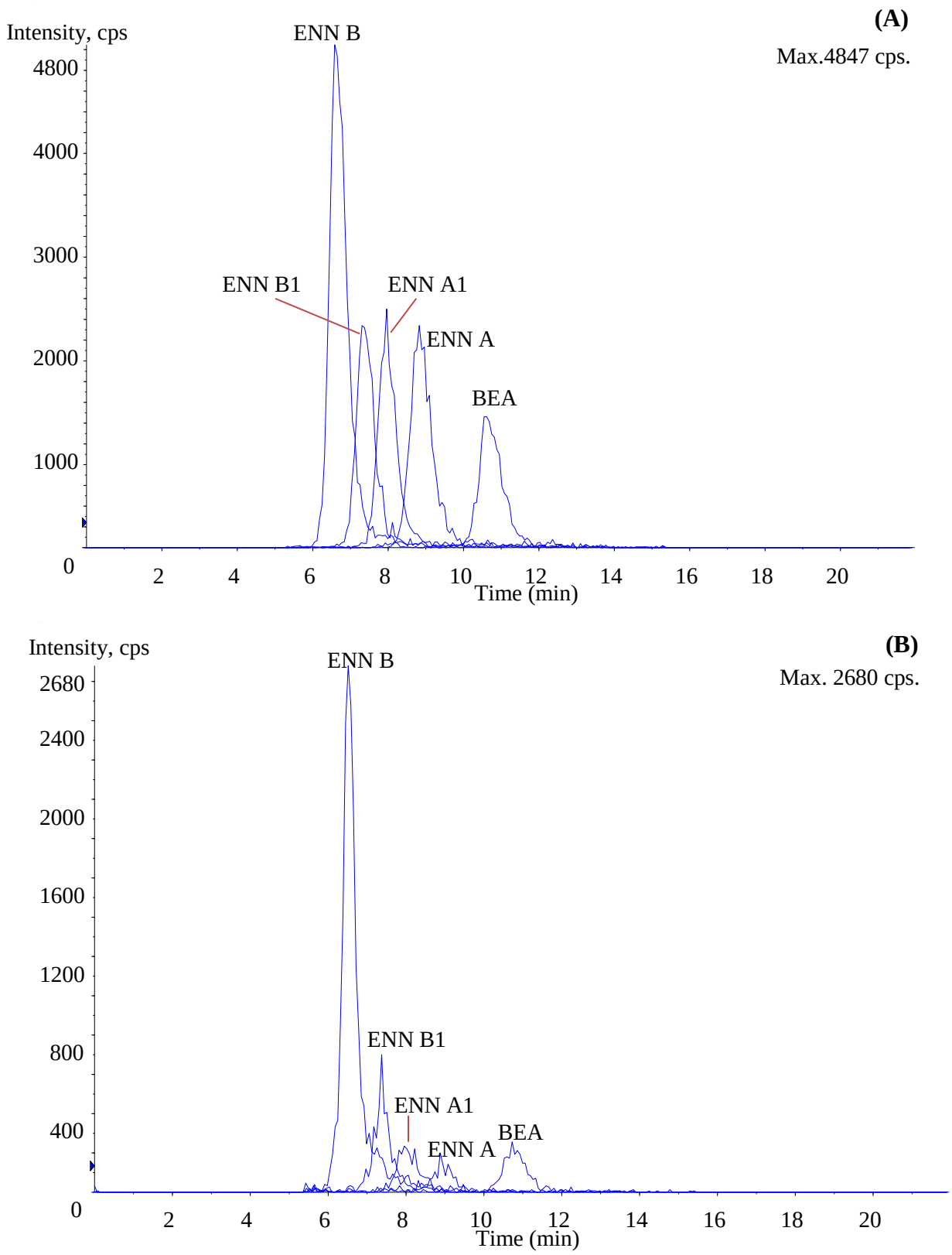


Figure 3-23. LC-MS/MS chromatograms of W1: **(A)** no SPE cloeanup; **(B)** MycoSep 225 cleanup

3.3.2 Method validation

Calibration and quantitation

Calibration curves were obtained by linear regression, showing good linearity within the chosen molar ratios (0.1–10) confirmed by the Mandel test. The response functions are as follows [y = n(A)/n(S), x = A(A)/A/S]: ENN A, y = 1.8692x – 0.0406 (R^2 = 0.9975); ENN A1, y = 1.4310x – 0.0821 (R^2 = 0.9984); ENN B, y = 1.5138x – 0.0674 (R^2 = 0.9958); ENN B1, y = 1.7618x – 0.1002 (R^2 = 0.9919); BEA, y = 0.9042x – 0.1627 (R^2 = 0.9971). Residual plots were drawn to examine the appropriateness of using linear regression, and all five plots showed random patterns. The response factors for ENNs are all above 1.4 and exceed the usual response factors around 1.0 for stable isotope dilution assays. However, multiple and regular tests of the calibration curve confirmed these unusual values. Therefore, additional isotope effects have to be assumed.

Limits of detection (LODs) and quantitation (LOQs)

LODs and LOQs were calculated according to Vogelgesang and Hädrich (1998), based on a calibration curve obtained from spiking experiments in a matrix free from the respective analyte. As shown in **Table 3-13**, the LODs are from 1.9 to 4.4 µg/kg, and LOQs are from 5.8 to 13.1 µg/kg.

Precision

The precision is given as inter-day (n = 3) and intra-day (n = 5) coefficients of variation (**Table 3-13**), varying between 1.35% and 8.61%.

Recovery

The recoveries determined with different spiking levels (20, 35, and 50 µg/kg) of each mycotoxin are given in **Table 3-13**.

Table 3-13. Validation data of the SIDA for ENNs and BEA (Synergi Polar RP 80A column)

	LOD	LOQ	Coefficients of variation		Recovery (3 spiking levels)		
	(µg/kg)	(µg/kg)	Inter-day (n = 3)	Intra-day (n = 5)	20 µg/kg	35 µg/kg	50 µg/kg
ENN A	3.9	11.5	1.36%	1.35%	98 ±7.6%	105 ±3.4%	107 ±7.2%
ENN A1	2.6	7.6	8.61%	6.31%	96 ±2.6%	102 ±4.8%	98 ±2.2%
ENN B	3.7	10.9	5.58%	7.21%	99 ±3.9%	100 ±3.1%	106 ±6.9%
ENN B1	1.9	5.8	4.09%	4.89%	105 ±5.1%	100 ±1.8%	104 ±1.9%
BEA	4.4	13.1	1.48%	1.41%	110 ±3.4%	109 ±1.0%	103 ±8.6%

3.3.3 Analysis of cereals and related food samples

A series of cereals and related food samples were analyzed for ENNs and BEA contamination using the SIDA developed. The results are summarized in **Table 3-14**. **Figure 3-24** presents the LC-MS/MS chromatograms of a barley sample.

Overall, the findings show high incidences of particularly ENN B and ENN B1. Except for rice, all the samples analyzed contain at least one of the five mycotoxins. The percentages of samples contaminated with ENNs A, A1, B, B1 and BEA were 16.9%, 52.3%, 87.7%, 83.1%, and 24.6%, respectively.

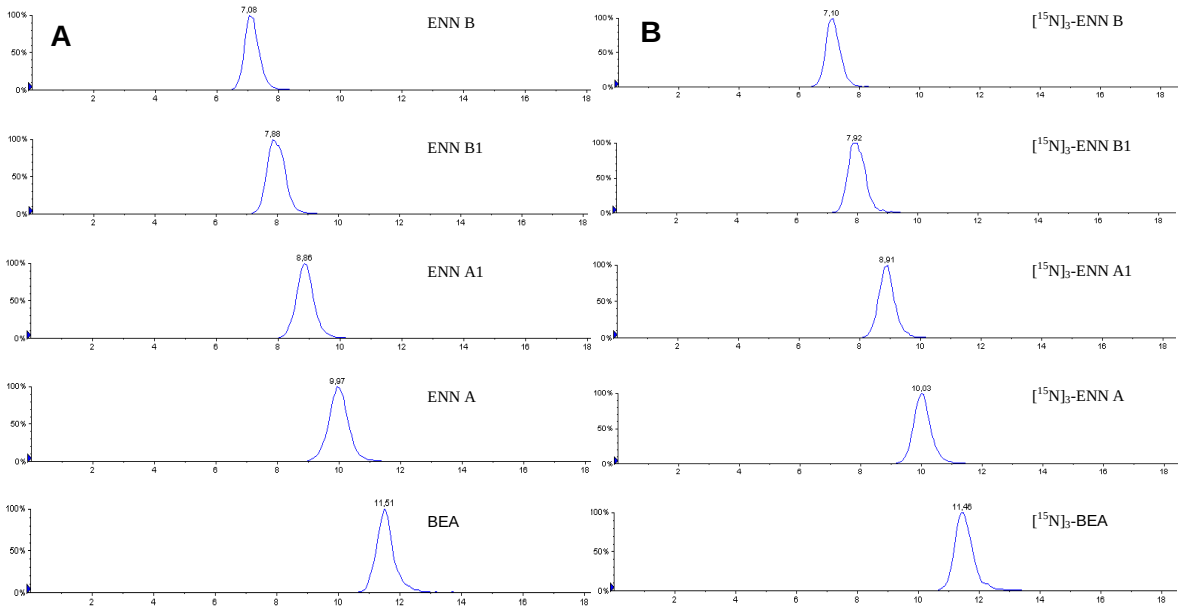


Figure 3-24. LC-MS/MS chromatograms of a barley malt sample: (**A**) analytes; (**B**) $^{15}N_3$-labeled standards

Table 3-14. Presence of ENNs and BEA in analyzed cereals and related food samples (μg/kg)

Samples	Number	ENN A			ENN A1			ENN B			ENN B1			BEA	
		Positive samples	Min-max[a]	Mean[b]	Positive samples	Min-max[a]	Mean[b]	Positive samples	Min-max[a]	Mean[b]	Positive samples	Min-max[a]	Mean[b]	Positive samples	Min-max[a]
barley malts	6	5	52-448	220	6	24-2721	1225	6	196-6998	3668	6	138-6762	3624	2	nq
wheat grains	6	5	nq-38	17	6	33-232	111	6	508-2125	1306	6	210-1066	658	2	nq
oat grains*	2	0	-	-	0	-	-	2	nq	5	2	nq	3	1	nq
rice grains	6	0	-	-	0	-	-	0	-	-	0	-	-	0	-
maize grains	2	0	-	-	0	-	-	2	nq	5	2	3-4	4	2	nq
maize grits*	2	0	-	-	0	-	-	1	11	6	-	-	-	0	-
maize flour	1	0	-	-	0	-	-	1	nq	5	1	nq	3	1	nq
wheat bread	5	0	-	-	3	nq	3	5	17-90	47	5	7-35	21	0	-
rye bread	5	0	-	-	3	nq-23	8	5	25-735	263	5	9-256	88	0	-
wheat flour	5	1	7	3	4	7-45	15	5	41-332	125	5	13-217	75	0	-
wheat flour*	5	0	-	-	4	nq-21	7	4	48-114	65	5	nq-74	33	1	nq
oat flakes	5	0	-	-	2	9-13	5	5	nq-94	42	4	nq-50	21	3	nq
oat flakes*	5	0	-	-	0	-	-	5	nq-62	24	3	nq-21	9	4	nq
spaghetti	5	0	-	-	4	nq-12	5	5	22-642	234	5	6-134	54	0	-
spaghetti*	5	0	-	-	2	nq	2	5	nq-68	25	5	nq-19	11	0	-

-, not detectable; nq, detected, but below limit of quantitation.

*organic samples, the rest of the samples were conventional.

[a]Min = minimum detected value; max = maximum detected value.

[b]Mean = mean value of all samples in the category, with not quantifiable and not detectable results considered as nq = ½(LOQ + LOD), - = ½(LOD).

3.4 Fate of ENNs and BEA during beer making

Three batches of barley grains were used to study the fate of ENNs and BEA during beer making. Batches QFc and QFa were artificially inoculated in field with *Fusarium culmorum* and *F. avenaceum*, respectively, and the control batch QC remained un-inoculated. The inoculation was carried out by Chair of Phytopathology, Technische Universität München (Hu et al., 2014).

3.4.1 Sample preparation and analysis

The SIDA of ENNs and BEA developed here was used to follow their fate during beer making. The method validation was updated as a different type of HPLC column was used. As shown in **Table 3-15**, the method reveals good recoveries (90–110%), precisions (CV = 0.9–5.5%), and sensitivities, with LODs and LOQs in range between 0.4–1.2 µg/kg and 1.2–3.5 µg/kg, respectively. Thus, the sensitivity should be sufficiently low according to previous reports on contamination of cereals with these toxins (Mahnine et al., 2011; Sørensen et al, 2008).

Table 3-15. Validation data of the SIDA for ENNs and BEA (YMC-Pack ProC18 column)

	LOD	LOQ	Precision (coefficients of variation)		Recovery (3 spiking levels)		
	(µg/kg)	(µg/kg)	Inter-day (n = 3)	Intra-day (n = 5)	5 µg/kg	15 µg/kg	20 µg/kg
ENN A	1.2	3.5	4.12%	3.47%	101 ±4.0%	100 ±4.7%	100 ±5.4%
ENN A1	0.4	1.2	1.36%	2.53%	99 ±6.6%	103 ±1.1%	104 ±0.4%
ENN B	0.8	2.2	1.23%	4.28%	102 ±5.3%	104 ±4.6%	105 ±2.1%
ENN B1	1.2	3.5	0.93%	3.68%	94 ±1.5%	99 ±4.3%	102 ±5.0%
BEA	0.8	2.4	5.52%	4.92%	99 ±3.9%	97 ±2.6%	94 ±2.6%

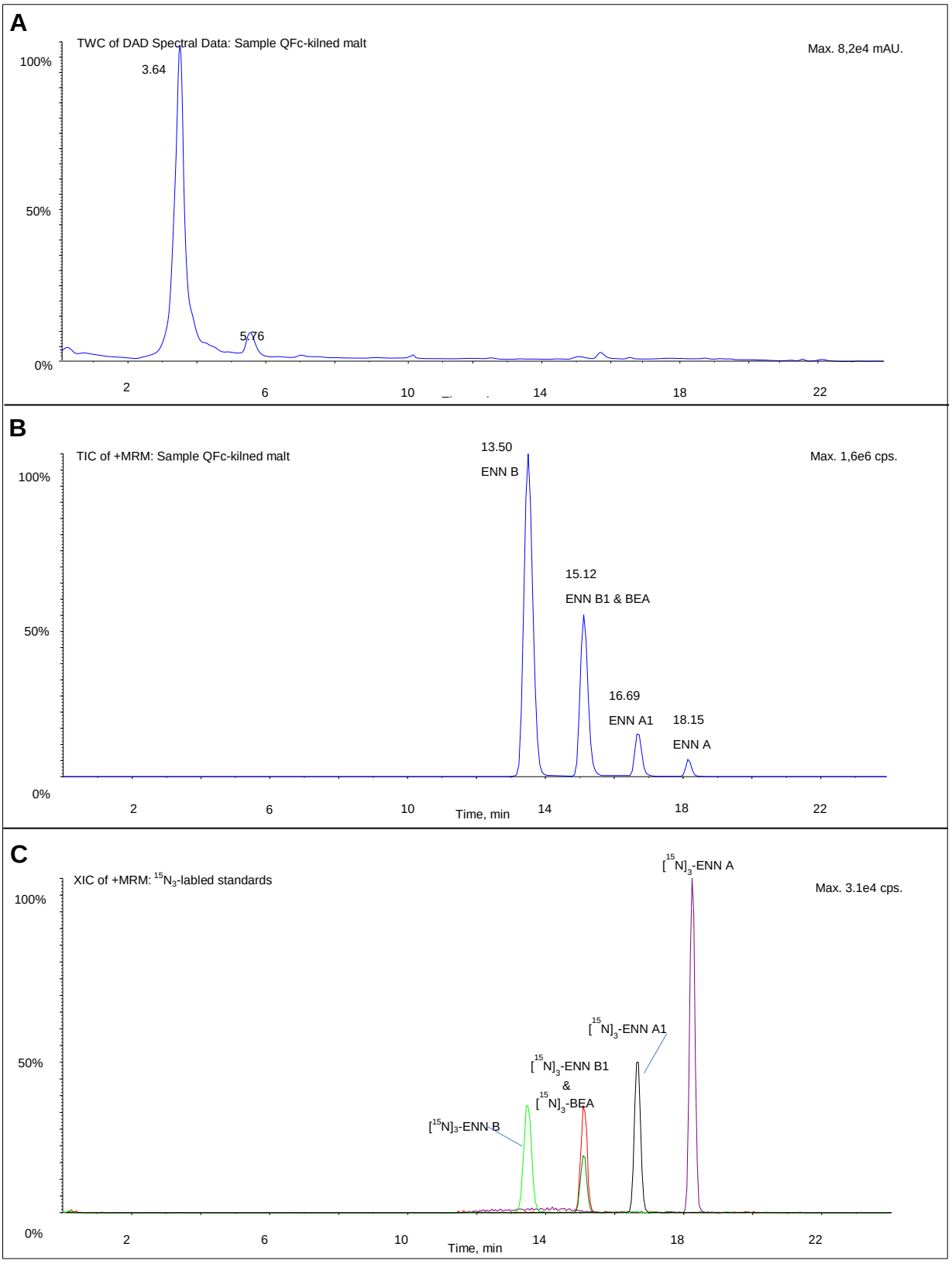
In this study, accuracy is achieved by the addition of isotope-labeled internal standards that compensate for losses and the matrix effects during MS/MS measurement. Nevertheless, due to the lack of extensive cleanup, it was still suspected that the sample extract might contaminate the mass spectrometer. To settle this question, an undiluted kilned malt (batch QFc) extract regarded as one of those loaded with most matrix interferences, was measured on the LC-MS/MS system combined with a Shimadzu PDA detector covering the wavelengths from 190 to 360 nm. As shown in **Figure 3-25**

(A), the major peaks, i.e. the unwanted contaminants, were eluted between 3 and 6 min, during which period the effluent from the column was not directed to the mass spectrometer but to the waste. Therefore, these contaminants would cause no harm to the mass spectrometer. Moreover, extracts of samples such as steeping water, wort and beer, which contained less matrix load, would pose an even smaller risk to the mass spectrometer.

Some samples contained more than 1 mg/kg ENNs, which would require an addition of more than 1 µg of the labeled standards to fall into the linear range of calibration. As these additions would consume too much of the stock of standards, an alternative approach had to be pursued. Due to reasons of homogeneity, the sample weight was not reduced, instead, the addition of labeled standards after sample extraction to an aliquot of the extract was tested. Its equivalence to the addition at the beginning of the extraction was demonstrated in a comparison experiment, as the coefficients of variance between the two methods were below 1.3 % for the ENNs under study.

As shown in **Figure 3-25 (B** & **C)**, the YMC-Pack ProC18 column used here renders narrow peaks and separates the ENNs well and, although the peak of BEA overlapped with that of ENN B1, they can be distinguished using their specific mass transitions.

Figure 3-25. The combined HPLC-DAD (**A**) and LC-MS/MS (**B** & **C**) chromatograms of a kilned malt (QFc) sample.



3.4.2 Behavior of ENNs and BEA during malting

The concentrations of ENNs and BEA in the barley grains, green malt, as well as first and second steeping water are given in **Table 3-16**, in addition, the total contents of each mycotoxin in green malt and steeping water are compared to those in the barley grains. The two steeping steps remove 23–38% of ENN B from the barley grains, while the reduction of ENNs A1 and B1 is less (2.5–22.5%). The ENN A and BEA in the steeping water are below the limits of detection.

The concentrations of ENNs and BEA in green malt, kilned malt, and rootlets are listed in **Table 3-17**, and the total amounts of each mycotoxin in the latter two fractions are compared to those in green malt. After kilning, only 41–72% of the ENNs and BEA originally present in green malt remain in kilned malt, 2.5–13.5% of ENNs and a higher percentage (14–28%) of BEA are removed from the kilned malt along with the discarded rootlets.

Table 3-16. Concentrations (µg/kg) of ENNs and BEA in the barley grains, green malt and first and second steeping water, and their contents compared to those in the barley grains (in total and in percentages)

QC	ENN A			ENN A1			ENN B			ENN B1			BEA		
		total			total			total			total			total	
	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%
barley	13.8	23.9	100.0%	883.3	1528	100.0%	5222	9034	100.0%	3515	6081	100.0%	9.9	17.1	100.0%
1st steeping	-	-	-	9.0	53.9	3.5%	367.6	2206	24.4%	105.3	632.0	10.4%	-	-	-
2nd steeping	-	-	-	nq	nq	nq	67.5	540.1	6.0%	23.9	191.0	3.1%	-	-	-
green malt	20.4	34.8*	145.8%	1474	2516*	164.6%	7973	13610*	150.6%	5967	10180*	167.5%	16.0	27.3*	159.5%
QFc	**ENN A**			**ENN A1**			**ENN B**			**ENN B1**			**BEA**		
		total			total			total			total			total	
	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%
barley	4.5	7.7	100.0%	252.9	431.7	100.0%	2949	5033	100.0%	1576	2690	100.0%	8.0	13.7	100.0%
1st steeping	-	-	-	nq	nq	nq	102.7	821.7	16.3%	22.2	177.4	6.6%	-	-	-
2nd steeping	-	-	-	4.0	31.6	7.3%	136.3	1090	21.7%	53.6	428.7	15.9%	-	-	-
green malt	12.2	20.6*	268.4%	877.8	1484*	343.7%	6052	10230*	203.2%	3540	5984*	222.4%	8.5	14.4	105.2%
QFa	**ENN A**			**ENN A1**			**ENN B**			**ENN B1**			**BEA**		
		total			total			total			total			total	
	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%
barley	38.8	67.7	100.0%	4046	7064	100.0%	119400	208400	100.0%	40690	71050	100.0%	14.8	25.8	100.0%
1st steeping	-	-	-	-	-	-	2863	22900	11.0%	105.5	844.3	1.2%	-	-	-
2nd steeping	-	-	-	40.5	323.7	4.6%	3038	24300	11.7%	113.2	906.0	1.3%	-	-	-
green malt	36.1	64.2	94.7%	3449	6131*	86.8%	117800	209400	100.5%	37480	66640	93.8%	14.5	25.8	99.8%

-, not detectable; nq, not quantifiable

*total content in green malt which was significantly different ($p < 0.05$) from that in barley

Table 3-17. Concentrations (µg/kg) of ENNs and BEA, and their contents in kilned malt and rootles compared to those in green malt (in total and in percentages)

QC	ENN A			ENN A1			ENN B			ENN B1			BEA		
		total			total			total			total			total	
	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%
green malt	20.4	34.8	100.0%	1474	2516	100.0%	7973	13600	100.0%	5967	10180	100.0%	16.0	27.3	100.0%
kilned malt	10.5	14.7*	42.3%	734.3	1030*	40.9%	5226	7327*	53.8%	3633*	5093*	50.0%	8.3	11.7*	43.2%
rootlets	37.1	2.3	6.7%	2150	135.5	5.4%	10300	648.9	4.8%	7756	488.6	4.8%	72.6	4.6	16.7%
QFc	ENN A			ENN A1			ENN B			ENN B1			BEA		
		total			total			total			total			total	
	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%
green malt	12.2	20.6	100.0%	877.8	1484	100.0%	6052	10230	100.0%	3540	5983	100.0%	8.5	14.3	100.0%
kilned malt	11.1	14.7*	71.4%	670.3	888.8*	59.9%	4690	6219*	60.8%	3211	4258*	71.2%	6.8	9.0*	62.8%
rootlets	17.1	1.5	7.3%	994.0	87.0	5.9%	2946	257.8	2.5%	2909	254.5	4.3%	46.3	4.1	28.2%
QFa	ENN A			ENN A1			ENN B			ENN B1			BEA		
		total			total			total			total			total	
	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%
green malt	36.1	64.2	100.0%	3449	6132	100.0%	117800	209400	100.0%	37480	66640	100.0%	14.5	25.8	100.0%
kilned malt	25.7	34.7*	54.1%	2915	3939*	64.2%	92690	125200*	59.8%	35460	47910*	71.9%	12.7	17.2*	66.6%
rootlets	95.5	8.7	13.5%	5635	512.8	8.4%	107300	9765	4.7%	44820	4079	6.1%	40.9	3.7	14.4%

*total content in kilned malt which was significantly different ($p < 0.05$) from that in green malt

3.4.3 Disposition of ENNs and BEA during brewing

The kilned malt was regarded as the starting point of the brewing process. In the beginning of brewing, it was ground into fine grits and extracted by water in the mashing procedure, after which the sweet wort was separated from the spent grains. As shown in **Table 3-18**, 64–98% of ENNs and 53–85% of BEA originally present in kilned malt are retained in spent grains, the sweet wort contains no more than 6% of ENNs and no detectable BEA.

In the following step, the sweet wort was boiled with hops, then the trub was precipitated and the wort was cooled. Although no ENN A is detectable in sweet wort, it is found in the trub, ranging from 1–8% of that in the kilned malt. Probably the ENN A in sweet wort is too diluted to be detected. The same is evident for BEA, as it is detected in the trub of QC and QFa in spite of the fact that no BEA is detectable in the respective sweet worts. No target mycotoxins are quantifiable in the cool wort of batch QC and no more than 1.6% of them are found in QFc and QFa.

The residues of ENNs and BEA in trub which exceed their LOQs account for 0.9–8.1% of those in kilned malt. Losses may have happened during sample preparation of the trub as it was heated at 80 ℃ for 12 h in an oven. Therefore, the true amounts of these mycotoxins in trub could be higher than detected.

In the next step of brewing, yeast was added into the cool wort to start the 6-day fermentation. At the end of fermentation, no ENNs A, A1, and B1 and BEA is detected in the green beer of QC, with ENN B being below LOQ. For QFc, no ENN A and BEA are found, with ENN A1 being below its LOQ and ENNs B and B1 are only 9 and 4 μg/kg, respectively. For QFa, no ENN A and BEA are detected, the concentration of ENN A1 decreases from 10.5 μg/kg in the cool wort to 6 μg/kg in the green beer, ENN B declines from 297 to 219 μg/kg, and ENN B1 drops from 121 to 61 μg/kg. Small fluctuations of the concentrations of ENNs B and B1 are found during the 6 days of fermentation, possibly due to adsorption of the mycotoxins by yeast or due to inhomogenous sampling.

Finally, the maturation and filtration of green beer see further decreases of ENNs A1, B and B1 in QFa, which have been treated with *Fusarium avenaceum* and is the only batch still containing ENNs above limits of quantitation. In the final beer, the concentration of ENN A1 in QFa declines to not detectable, ENNs B and B1 decline to 74 (i.e. by 66% after maturation and filtration) and 14 μg/kg (i.e. by 77%

after maturation and filtration), respectively. The carryovers of ENNs B and B1 from the initial barley grains to final beer are 0.2% and 0.1%, respectively.

The yeast sediment after filtration of beer contains up to 1045 µg/kg (by dry weight) of ENNs.

In hop, the ENN B level is 7 µg/kg, the other ENNs and BEA are either not detected or below limits of quantitation.

Table 3-18. Concentrations (µg/kg) of ENNs and BEA in different stages of brewing, and their contents remaining to kilned malt (in total and in percentages)

QC	ENN A			ENN A1			ENN B			ENN B1			BEA		
		total			total			total			total			total	
	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%
kilned malt	10.5	14.7	100.0%	734.3	1030	100.0%	5226	7327	100.0%	3633	5093	100.0%	8.3	11.6	100.0%
spent grains	33.3	13.9	94.8%	1905	796.1	77.3%	15530	6493	88.6%	10030	4194	82.3%	21.4	8.9	76.9%
sweet wort	-	-	-	nq	nq	nq	36.9	325.5	4.4%	12.6	111.1	2.2%	-	-	-
trub	31.6	1.2	8.1%	250.6	9.5	0.9%	7944	301.9	4.1%	3117	118.4	2.3%	7.8	0.3	2.5%
cool wort	-	-	-	-	-	-	nq	-	-	-	-	-	-	-	-

QFc	ENN A			ENN A1			ENN B			ENN B1			BEA		
		total			total			total			total			total	
	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%
kilned malt	11.1	14.7	100.0%	670.3	888.8	100.0%	4690	6219	100.0%	3211	4258	100.0%	6.8	9.0	100.0%
spent grains	24.8	11.9	80.7%	1420	681.5	76.7%	12510	6003	96.5%	7046	3382	79.4%	16.0	7.7	85.2%
sweet wort	-			3.7	31.9	3.6%	44.0	379.1	6.1%	28.7	247.3	5.8%	-	-	-
trub	3.8	0.2	1.4%	304.1	16.1	1.8%	5782	306.5	4.9%	2743	145.4	3.4%	-	-	-
cool wort	-			-	-	-	13.9	90.0	1.4%	8.7	56.4	1.3%	-	-	-

QFa	ENN A			ENN A1			ENN B			ENN B1			BEA		
		total			total			total			total			total	
	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%
kilned malt	25.7	34.7	100.0%	2915	3939	100.0%	92690	125200	100.0%	35460	47910	100.0%	12.7	17.2	100.0%
spent grains	83.0	34.0	98.0%	6140	2518	63.9%	251500	103100	82.3%	104200	42700	89.1%	22.3	9.1	53.3%
sweet wort	-	-	-	27.7	241	6.1%	713.8	6220	5.0%	331.8	2891	6.0%	-	-	-
trub	11.2	0.6	1.8%	1626	91.1	2.3%	43270	2423	1.9%	21400	1199	2.5%	21.5	1.2	7.0%
cool wort	-	-	-	10.5	64.8	1.6%	297.4	1836	1.5%	121.3	748.7	1.6%	-	-	-

-, not detectable; nq, not quantifiable

3.5 Effect of sourdough bread making on ENNs and BEA

3.5.1 Milling

The wheat and rye grains are both naturally contaminated with ENN B and ENN B1. The distribution of the ENNs within the two milling fractions bran and flour is significantly uneven. The concentrations of ENN B and ENN B1 in the wheat flour fraction are 105 and 110 µg/kg, respectively, while those in the wheat bran fraction are 1132 and 864 µg/kg, respectively. The contamination levels in the latter are about 7–10 times higher than those in the former. In the rye flour, the concentrations of ENN B and ENN B1 are 24 and 11 µg/kg, respectively, while in the rye bran, the concentrations are about 3 times higher (92 and 43 µg/kg of ENN B and ENN B1, respectively). As the weight ratio between rye flour and bran is approximately 50:50, 81–82% of ENN B and ENN B1 go to the bran fraction.

In the subsequent bread making process, wheat flour was used as starting material, while rye bran had to be mixed back to rye flour to produce wholegrain rye flour, otherwise the concentrations of the ENNs would have been too low to follow their fate during bread making.

3.5.2 Micro-sourdough preparation

As shown in **Figure 3-26**, after 24 h of sourdough fermentation at 30 ℃, the ENN B and ENN B1 in the flour are reduced by 7–10%, while the reduction of the artificially added BEA is not significant ($p < 0.05$). In comparison, the fermentation at 40 ℃ for 24 h leads to 17–19% reduction of the ENNs, and a 10% reduction of BEA. Apparently, the higher fermentation temperature is more effective in removing ENNs and BEA, which are possibly less stable at higher temperatures.

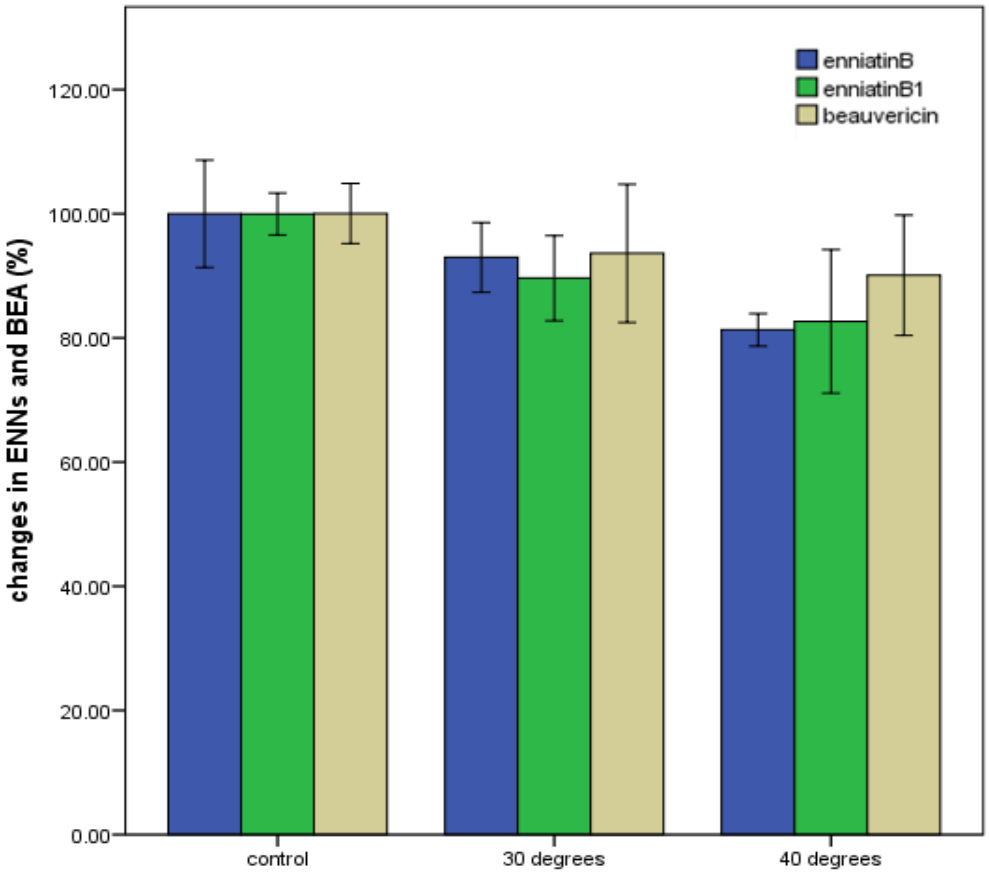


Figure 3-26. Reduction of ENNs and BEA during micro sourdough preparation

3.5.3 Behavior of ENNs and BEA during sourdough bread making

The changes of ENNs and BEA during different stages of sourdough bread making are summarized in **Table 3-19** and **3-20**.

For ENN B and ENN B1, homogeneity of variance was established by Levene's test of homogeneity of variance, and ANOVA was followed by LSD post hoc test. Because the sourdough starter was highly contaminated with ENN B (384 µg/kg) and ENN B1 (325 µg/kg), and 3 g of sourdough starter was added to 147 g of flour, the levels of the ENNs in flour was adjusted on a 3/150 basis.

No significant ($p < 0.05$) changes of ENNs occur during kneading, except for ENN B in case of rye sourdough baking where a 4% reduction is found to be significant.

Between the rested dough (10 min at 30 °C) and kneaded dough, no significant change of ENNs is observed. After proofing at 30 °C for 45 min, the ENNs are reduced by 13–19% compared to their original levels in respective flour.

In comparison to normal proofing at 30 ℃, proofing at 50 ℃ for 45 min reduces 16.5–28% of the ENNs compared to their concentrations in flour. The difference between the two types of proofed doughs is significant ($p < 0.05$) in case of rye sourdough baking, but not in case of wheat sourdough baking.

As for BEA, Levene's test of homogeneity of variance showed that the variance is unequal. Therefore, the Tamhane post hoc test was applied and generally no significant difference was found among the flour and the series of dough samples, with the exception between flour and rested dough in case of wheat sourdough bread making, and between flour and proofed dough in case of rye sourdough bread making, each revealing a 6% reduction. None of the other types of dough is found significantly different to flour, although fluctuations of BEA are observed during the dough preparation procedures. In general, the BEA content is not affected by the dough kneading and fermentation processes, which is quite different from ENN B and ENN B1.

For both wheat and rye sourdough bread, the final baking procedure at 200 ℃ for 25 min remove 9–28% of ENNs and BEA from proofed dough, with the exception of BEA in crumb in case of rye sourdough bread making, where the difference is not significant. For ENN B and ENN B1, stronger reductions are found in crust than in crumb (5% and 9%, respectively) in case of rye sourdough bread making. However, in case of wheat sourdough bread, the difference between crumb and crust is not significant.

Overall, the whole sourdough bread making process reduces ENN B, ENN B1 and BEA by 25–41% from the starting materials (flour and sourdough starter), except for BEA in crumb in case of rye sourdough baking.

Table 3-19. Changes of ENNs and BEA during sourdough bread making with wheat flour

	ENN B		ENN B1		BEA	
	µg/kg[1]	retention[2]	µg/kg[1]	retention[2]	µg/kg[1]	retention[2]
flour	111 ±2.8 a	100.0%	114 ±2.8 a	100.0%	86 ±0.6	100.0%
kd	102 ±6.4 ab	92.3%	107 ±4.6 a	94.1%	83 ±4.6	96.6%
rd	101 ±7.3 bc	91.2%	109 ±4.5 a	95.8%	81 ±0.8	94.2%
pd	97 ±3.3 bc	87.2%	94 ±5.8 b	82.2%	75 ±1.7	87.0%
pd50	92 ±5.5 c	83.5%	87 ±3.2 bc	75.8%	80 ±2.7	93.7%
crumb	80 ±5.2 d	72.5%	83 ±5.3 cd	72.9%	64 ±1.1	74.7%
crust	74 ±2.0 d	66.6%	75 ±9.4 d	65.5%	56 ±2.2	65.5%

kd - kneaded dough; rd - rested dough; pd - proofed dough (at 30 ℃); pd50: proofed dough (at 50 ℃)

[1] all the concentrations are adjusted on the basis of flour (i.e. µg/kg flour equivalent)

[2] percentage of recovered total amount relative to the initial amount in flour

Values of different letters in the same column are significantly different ($p < 0.05$).

Table 3-20. Changes of ENNs and BEA during sourdough bread making with wholegrain rye flour

	ENN B		ENN B1		BEA	
	µg/kg[1]	retention[2]	µg/kg[1]	retention[2]	µg/kg[1]	retention[2]
flour	64 ±0.4 a	100.0%	33 ±0.5 a	100.0%	86 ±0.6	100.0%
kd	62 ±1.1 b	95.8%	32 ±0.6 ab	97.5%	84 ±1.3	97.8%
rd	59 ±1.5 b	92.1%	30 ±2.4 bc	91.7%	88 ±3.6	102.3%
pd	52 ±1.4 c	81.0%	28 ±1.8 c	86.2%	81 ±0.6	94.1%
pd50	46 ±1.2 d	72.0%	24 ±1.4 de	74.1%	85 ±3.3	99.2%
crumb	43 ±1.5 e	67.1%	22 ±0.6 e	67.5%	68 ±3.3	79.7%
crust	40 ±2.3 f	62.5%	19 ±0.6 f	58.7%	60 ±2.1	70.2%

kd - kneaded dough; rd - rested dough; pd - proofed dough (at 30 ℃); pd50: proofed dough (at 50 ℃)

[1] all the concentrations are adjusted on the basis of respective flour (i.e. µg/kg flour equivalent)

[2] percentage of recovered total amount relative to the initial amount in flour

Values of different letters in the same column are significantly different ($p < 0.05$).

3.6 Occurrence of ENNs and BEA in Chinese medicinal herbs

3.6.1 Method validation

A blank mixture sample made from lotus leaf, chrysanthemum flower, kudzuvine root, and black seasame, all of which were free of ENNs and BEA, was used for method validation. Mean recoveries of the blank mixture sample spiked with $^{15}N_3$-labeled ENNs and BEA at levels of 2–20 μg/kg are 93–109% for ENNs and 93–101% for BEA, with relative standard deviations of 2.1–8.7% and 2.5–5.6% for ENNs and BEA, respectively. The LODs are 0.8 μg/kg (ENN A), 1.1 μg/kg (ENN A1), 1.0 μg/kg (ENN B), 1.1 μg/kg (ENN B1), and 1.2 μg/kg (BEA). The LOQs are 2.5 μg/kg (ENN A), 3.4 μg/kg (ENN A1), 2.8 μg/kg (ENN B), 3.2 μg/kg (ENN B1), and 3.7 μg/kg (BEA). Inter-day (n = 3) precision calculated as coefficients of variation are 2.8–7.1% for ENNs and 5.8% for BEA. Intra-day (n = 5) precision given in coefficients of variation are 3.5–6.3% for ENNs and 4.7% for BEA. Samples with contamination levels above LODs are considered to be positive, concentrations between LOD and LOQ are considered as the mean of LOD and LOQ.

3.6.2 Occurrence of ENNs and BEA in medicinal herbs

Of all the 60 medicinal herbs analyzed for the presence of ENNs and BEA, 15 samples are found to be contaminated with at least one of these mycotoxins, the frequency of contamination is 25%. The contamination levels of ENNs and BEA in positive samples are given in **Table 3-21**.

The mean contamination levels of positive samples are 28.9, 28.4, 32.0, 3.9, and 33.0 μg/kg for ENN A, ENN A1, ENN B, ENN B1, and BEA, respectively. The maximum levels are 355 μg/kg (ENN A), 253 μg/kg (ENN A1), 290 μg/kg (ENN B), 40.2 μg/kg (ENN B1), and 125 μg/kg (BEA). Ginger is detected with the highest levels of ENN A and ENN A1, cogongrass rhizome contains the highest levels of ENN B and BEA, and the highest level of ENN B1 is found in longstamen onion bulb.

BEA is the most frequently detected single mycotoxin in this study, with 12 positive samples, i.e. 20% of all examined samples. The incidences of ENNs are lower, with ENN B being most prevalent and detectable in 7 samples of all, i.e. 11.7 % and ENN A1 and B1 being least prevalent in 4 samples, i.e. 6.7 % of all samples.

As ginger is also a commonly used herb in Europe, 6 further ginger samples obtained from Bavarian markets were analyzed. The results in **Table 3-22** reveal common occurrence of ENNs and BEA in the dried ginger samples, with BEA being the predominant mycotoxin, while the ENNs do not reach the levels found in the Chinese sample. Interestingly, none of the mycotoxins under study is detectable in the fresh ginger sample, which points to the assumption that the contamination or production of these mycotoxins might have occurred during drying of ginger.

Considering the total amounts of the five mycotoxins in single samples, values between 2.5 and 751 µg/kg are found. The mean total amount in positive samples is 126 µg/kg. The maximum total amount of five mycotoxins is again found with ginger, and the second most highly contaminated is cogongrass rhizome, with a total amount of 624 µg/kg.

Regarding the co-occurrence of the ENNs and BEA, 6 samples are contaminated with more than three of these mycotoxins, i.e. 40% of the positive samples, or 10% of all samples.

Table 3-21. Contamination levels (µg/kg) of ENNs and BEA in positive herb samples

Name	ENN A	ENN A1	ENN B	ENN B1	BEA
Mint leaf	-	-	-	-	nq
Licorice root	nq	-	nq	-	11.6
Black pepper	-	-	-	-	6.0
Wrinkled giant hyssop herb	-	-	-	-	99.4
Ginger	355	253	125	-	19.0
Platycodon root	-	-	3.9	nq	-
Chinese quince fruit	-	-	-	-	4.6
Mulberry leaf	-	-	-	-	29.9
Spina date seed	-	-	-	-	nq
Smoked plum	3.4	-	-	-	-
Bitter apricot seed	-	nq	21.8	13.4	-
Cogongrass rhizome	59.0	150	290	-	125
Longstamen onion bulb	14.8	21.7	29.5	40.2	60.5
Bitter cardamom	-	-	-	-	45.7
Heartleaf houttuynia herb	-	-	7.7	nq	88.8

-, below LOD; nq, between LOD and LOQ.

Table 3-22. Contamination levels (µg/kg) of ENNs and BEA in ginger samples from Bavaria

Name	ENN A	ENN A1	ENN B	ENN B1	BEA
Dried ginger spice 1	22.4	20.6	nq	11.5	100.7
Dried ginger spice 2	90.2	65.0	6.2	30.9	117.9
Dried ginger spice 3	-	-	3.7	-	73.7
Dried ginger herb 1 (for infusions)	-	-	6.0	6.4	59.2
Dried ginger herb 2 (for infusions)	-	-	-	-	29.3
Fresh ginger root	-	-	-	-	-

-, below LOD; nq, between LOD and LOQ.

4. Discussion

4.1 Biosynthesis of $^{15}N_3$-labeled ENNs and BEA

In this study, $^{15}N_3$-labeled ENNs and BEA were produced by cultivating the mycotoxin-producing fungi in culture medium containing $Na^{15}NO_3$. In previous reports, similar protocols were applied to produce fungal peptides from culture medium containing $K^{15}NO_3$ (Yee & O'Neil , 1992) and chitin from culture medium containing $(^{15}NH_4)_2SO_4$ (Watson et al., 2009).

Using $Na^{15}NO_3$ instead of normal $Na^{14}NO_3$ in the culture medium, 430 µg ($[^{15}N]_3$-ENN A), 450 µg ($[^{15}N]_3$-ENN A1), 100 µg ($[^{15}N]_3$-ENN B), and 210 µg ($[^{15}N]_3$-ENN B1) were isolated from the culture of a *Fusarium sambucinum* strain. Given that a total amount of 1 g of $Na^{15}NO_3$ was used in the culture medium, and that the cost of $Na^{15}NO_3$ was US$ 71.5 (about € 54) per gram, this method proved to be very cost-effective for the production of isotope labeled ENNs. Similarly, 1460 µg of $[^{15}N]_3$-BEA were isolated from the culture medium (also using 1 g of $Na^{15}NO_3$) of a *Fusarium fujikuroi* strain.

Most of the isotope labeled mycotoxins available on the market are ^{13}C-labeled ones. In comparison to the incorporation of ^{15}N into the ENNs and BEA molecules, the incorporation of ^{13}C would be much more expensive in case of using the modified Czapek-Dox liquid minimal medium, as a total of 15 g of $^{13}C_6$-glucose would be needed, which would cost about € 3000.

4.2 SIDAs of ENNs and BEA in cereals and related food

4.2.1 Method validation

Limits of detection (LODs) and quantitation (LOQs)

With LODs of 1.9–4.4 µg/kg, and LOQs of 5.8–13.1 µg/kg, the SIDA presented here is similarly sensitive with the methods presented by Sørensen et al. (2008) and Uhlig et al. (2004). Compared to the methods reported by Mahnine et al. (2011) and Meca et al. (2010c), the present assay is more sensitive by two orders of magnitude. Compared to the method by Pamel et al. (2010), the present assay is five times more sensitive. In contrast, two further assays (Jestoi et al., 2009; Sewram et al., 1999) were reported to be approximately ten times more sensitive than the present one. However, the determination of LODs was not clear in both reports. Besides, the sample weight applied was 5-fold (Jestoi et al., 2009) and 20-fold (Sewram et al., 1999) as high as that in the present one, respectively.

Precision

With inter-day (n = 3) and intra-day (n = 5) coefficients of variation varying between 1.35% and 8.61%, the SIDA presented here is similarly precise as the method reported by Mahnine et al. (2011). In contrast, the method of Pamel et al. (2011) was less precise as the relative standard deviations ranged between 8% and 49%.

Recovery

All the recoveries fell in the range between 90% and 120%, with low standard deviations. In comparison, all the other methods (Sewram et al., 1999; Uhlig et al., 2004; Sørensen et al., 2008; Jestoi et al., 2009; Meca et al., 2010c; Pamel et al., 2010; Mahnine et al., 2011) revealed recoveries as low as or far below 85% for at least one of the ENNs and BEA.

4.2.2 Analysis of cereals and related food samples

A total of 65 cereals and related food samples were analyzed by SIDAs. The occurrence and concentrations of ENNs were in a distinct ratio (ENN B > ENN B1 > ENN A1 > ENN A), which was in accordance with previous investigations on Norwegian grains and Danish maize (Uhlig et al., 2006; Sørensen, et al., 2008). Wheat grains and barley malts were the most severely contaminated with ENNs, and the highest levels of all four ENNs were detected in barley malts, with the amount of ENN B and ENN B1 reaching 7000 and 6760 μg/kg, respectively. The contents of ENNs in other food samples were significantly lower, ranging from not detectable to 735 μg/kg. Earlier studies have reported the presence of ENNs in a variety of food samples, including wheat, barley, oat, maize, and cereal based products with the levels of ENNs covering a wide range between below 0.6 μg/kg and 814 mg/kg (Mahnine et al., 2011; Sørensen, et al., 2008; Uhlig et al., 2006; Meca et al., 2010c; Jestoi et al., 2004). Therefore, the present results of ENNs levels (from less than 5.8 μg/kg to 7000 μg/kg) fell within the range of previous reports, but the maximum level was considerably lower.

Interestingly, none of the 65 samples contained BEA above limit of quantitation (13.1 μg/kg), whereas other groups have reported cereals from Spain (Meca et al., 2010c), Poland (Logrieco et al., 1993), and Italy (Ritieni et al., 1997) with BEA levels up to 11.8, 60, and 520 mg/kg, respectively.

In addition, three sets of organic and conventional cereal products (wheat flour, oat flakes, and spaghetti) were compared for their contamination of ENNs and BEA. The organic products were found to be less contaminated with ENNs in average, their maximum levels were also lower. The frequency of BEA in organic products was slightly higher than that in conventional ones. However, due to the negligibly low amount of BEA in all samples, this would not mean the organic products were a hazard to the consumers.

4.3 Fate of ENNs and BEA during beer making

4.3.1 Behavior of fungal species, ENNs and BEA during malting

Three batches of barley grains were used as the starting materials for malting, batch QC was the control group, batch QFc was artificially inoculated with *Fusarium culmorum*, and batch QFa was artificially inoculated with *Fusarium avenaceum*. Although QC was not artificially inoculated with *Fusarium* strains, quantitative polymerase chain reaction (qPCR) of the fungal DNA revealed that it was naturally contaminated with *F. avenaceum*, and its contamination level of ENNs was higher than that of QFc (Hu et al., 2014), which was artificially inoculated with *F. culmorum* and the latter is known to be no ENN producer (Desjardins, 2006, p.260). As expected, QFa presented the highest contamination level of the mycotoxins and *F. avenaceum* was confirmed to be a very potent producer of ENNs. However, the strain used in this study did not produce BEA in a similar dimension as the ENNs. Logrieco et al. (2002) have reported a similar situation, when 13 strains of *F. avenaceum* were cultured on rice, 3 strains were found not to produce BEA, meanwhile these 3 strains produced ENNs A1, B, and B1 with concentrations up to 860 µg/g.

During steeping, unlike DON and 15- and 3-acetyl-DON, which were largely reduced by steeping to below quantitation limits (Schwarz, Casper, & Beattie, 1995; Lancova et al., 2008), the major part of ENNs and BEA remained in the barley grains, obviously due to their low water solubility. The findings in this study were partly contrary to those of Vaclavikova et al. (2013), who observed that the levels of ENNs A and A1 decreased to 10–20% of their initial levels in the barley used as raw material. According to the data delivered by the latter authors, steeping must have reduced the mycotoxins substantially. Unfortunately, no analyses of the steeping water were presented to support this conclusion, but in the present study, analyses of the steeping water and the material in the different malting stages contravene this hypothesis.

Similar to the reports of Schwarz et al. (1995) and Lancova et al. (2008), production of mycotoxins occurred in this study during germination. For batch QC, the amount of ENNs and BEA in green malt increased by about 50%, correspondingly, qPCR revealed an increase of *F. avenaceum* DNA of about 50% (Hu et al., 2014). The increases of ENNs were much higher for batch QFc, which were between 103% and 244%, again paralleled by an increase of *F. avenaceum* DNA of over 300% (Hu et al.,

2014), although BEA did not change significantly ($p < 0.05$). On the contrary, for QFa, there was a slight decrease of ENN A1, while the rest four mycotoxins did not change significantly ($p < 0.05$). For this batch, however, *F. avenaceum* DNA still increased about 4.5 fold (Hu et al., 2014), obviously, *F. avenaceum* was not able to produce higher mycotoxin levels or stopped production when the amounts reached these high levels.

During the kilning stage, 21–54% of ENNs and 9–40% of BEA were eliminated, possibly by thermal or biological degradation. Meca et al. (2012) reported that BEA was degraded by 20–90% after being heated at 160, 180, and 200 °C for 20 min, respectively. However, the kilning of green malt was carried out at lower temperatures (between 50 and 80 °C), although for a longer time (in total, 23 h). Thus, the thermal degradation of BEA cannot be substantiated by the findings of the latter authors, but, nonetheless, it is a likely pathway. With regard to the thermal degradation of ENNs, no detailed information was reported as far as I know. To shed light on this phenomenon, a simulation experiment was carried out, 100 ng of ENN B and BEA each were added to 1 g of a barley grain sample originally containing none of the mycotoxins above their LOQ. Then, the sample was heated in an oven with the same heating times and temperatures used for kilning. The losses of ENN B and BEA after the treatment were 29% and 16%, respectively, which fell within the range of the losses found during kilning and confirmed thermal degradation to be the main cause for the decrease of the toxins. These results are contradictory to those recently reported by Vaclavikova et al. (2013), who partly observed an increase of some ENNs during kilning. Unfortunately, no explanations for this unexpected finding were presented. Concerning the effect of discarding the mycotoxins with rootlets, the data of the latter authors cannot be evaluated as, unlike the present data, only the concentrations but no total amounts or balances were given.

Biological degradation of ENNs and BEA could be another possible explanation. As demonstrated by Abrunhosa et al. (2002), a number of *Aspergillus* fungi were able to degrade more than 80% of ochratoxin A in culture medium, among which were some producers of ochratoxin A. The same group later isolated the enzyme responsible for the degradation process (Abrunhosa & Venâncio, 2007). Therefore, the *Fusarium* fungi or enzymes that were active during the kilning stage might have played a role in the degradation of ENNs and BEA. To fully understand the mechanisms for the degradation of ENNs and BEA during kilning, further researches would be necessary. In comparison, Lancova et

al. (2008) reported that kilning did not change the levels of the trichothecenes DON and acetylated DONs, neither did thermal degradation as they are stable up to 120 ℃. In contrast to this, a study on the fate of five triazole fungicides during beer making by Navarro et al. (2011) revealed that kilning lowered their contents by 2.5–9.5%.

4.3.2 Disposition of ENNs and BEA during brewing

After mashing of the kilned malt and separation of the sweet wort from spent grains, 64–98% of the ENNs originally present in kilned malt was retained in spent grains, the percentages were in accordance with those of Vaclavikova et al. (2013), who reported 64–91% of the ENNs to remain in spent grains. On the contrary, according to previous researches, the highly water-soluble DON was either not detected or detected only in traces in spent grains (Kostelanska et al., 2011; Lancova et al., 2008; Schwarz et al., 1995) and, most of this toxin was transferred into sweet wort. In a study of the fate of 312 pesticides during beer brewing, Inoue et al. (2011) observed that the more hydrophobic compounds were adsorbed more rapidly onto spent grains. Therefore, given their low water solubility, the high contamination levels of ENNs and BEA in spent grains were not unexpected. These spent grains could pose possible risks to animals, as they are used as a buffer, forage or concentrate replacer in feed for ruminant animals (Navarro et al., 2005).

During the fermentation of the wort into green beer, decreases were found for ENNs, whereas BEA was already below limit of detection in the wort. Similarly, decreases of ochratoxin A and fumonisins were observed by Scott et al. (1995) when added to wort and fermented for 8 days by *Saccharomyces cerevisiae*, losses were between 2% and 28%. Strains of *Saccharomyces cerevisiae* were also reported to degrade patulin during the alcoholic fermentation of apple juice (Moss & Long, 2002).

Finally, the maturation and filtration of the green beer led to further decreases of ENNs in batch QFa, where they were still detectable, with ENNs B and B1 being reduced by 66% and 77%, respectively. In comparison, in the report by Navarro et al. (2005), the fungicides myclobutanil and propiconazole were lessened by 50% and 25%, respectively, after maturation and filtration, and the authors suggested surface adsorption as a probable cause. Scott et al. (1995) also reported up to 21% of ochratoxin A taken up by yeast during fermentation of wort. On the basis of this notion, the yeast sediment after filtration of beer was analyzed and up to 1045 μg/kg (by dry weight) of ENNs were detected. However,

as yeast was partially lost during filtration, its total amount was unknown. Therefore, the finding can only confirm that some of the ENNs were adsorbed by yeast, but the exact percentage cannot be calculated. As yeast residue is used as raw material for feeds and foods, further attention has to be drawn to occurrence of ENNs in respective samples. Moreover, unfiltered beer could contain detectable amounts of these mycotoxins.

The levels of ENNs and BEA in hop were low, in any case, their origin from hop could be neglected, as only 8 g were used for each batch.

4.4 Effect of sourdough bread making on ENNs and BEA

4.4.1 Milling

Based on the weight ratio between wheat flour and bran (77:23), as well as the concentrations of ENNs in the two fractions, 70–76% of ENN B and ENN B1 were removed with wheat bran. The percentages were in agreement with those of Vaclavikova et al. (2013), who found that 71–79% of ENN B and ENN B1 were removed during the milling process of white flour.

Similar to ENNs, the uneven distribution of the major *Fusarium* mycotoxin DON in milling fractions of wheat has been reported in previous researches. The highest concentration was often found in the outer layers of the wheat (eg. bran and shorts), and the lowest in the flour, the concentration in the former varied from 4–15 folds to that in the latter (Abbas et al., 1985; Simsek et al., 2012). Young et al. (1984) reported that DON was produced at the site of fungal growth rather than being transported from the kernel surface to the interior. This might also explain for the higher ENNs levels in the bran.

4.4.2 Behavior of ENNs and BEA during sourdough bread making

During kneading of the dough, generally no significant ($p < 0.05$) changes of ENNs occurred. The effect of kneading on mycotoxins was rarely studied on its own, in the two previous reports regarding DON, the results were not consistent. Lancova et al. (2008) reported that the levels of DON were 21–40% higher in kneaded dough than in flour, while Kostelanska et al. (2011) using similar baking ingredients observed only a slight increase of DON after kneading, but it is unknown whether this increase was significant due to lack of statistical analysis.

After fermentation (resting for 10 min, followed by proofing for 45 min, both at 30 ℃), the ENNs were reduced by 13–19% compared to their original levels in respective flour. In a study on changes of ochratoxin A in wheat flour (Valle-Algarra et al., 2009) under similar conditions of dough fermentation (29–30 ℃ for 1 h), reductions between 29.8–33.5% were observed. However, in the only report on ENNs during baking, Vaclavikova et al. (2013) observed inexplicable changes, when ENN B and ENN B1 were first reduced significantly after fermentation for 50 min at 30 ℃, then increased significantly after proofing for 45 min (Lancova et al., 2008) at the same temperature. The ENNs in

the proofed dough varied widely from approximately 40% to 80% of their original contents in flour. Apparently, like the many conflicting results concerning changes of DON concentration during baking (Simsek et al., 2012), the behavior of ENNs are also case-dependent, the shorter fermentation time in combination with the use of sourdough in the present study might have attributed to the difference between the study of Vaclavikova et al. (2013) and the present one.

In comparison to normal proofing at 30 ℃, proofing at 50 ℃ reduced significantly ($p < 0.05$) more of the ENNs in case of rye sourdough baking, which suggests that the higher fermentation temperature is more effective in reducing ENNs. Similar trends were observed for DON during French bread making by Samar et al.(2001), who reported that after dough fermentation at 50 ℃ for 40 min, the DON level was 41% lower than that in the flour, while fermentation at 30 ℃ either for 30 min or 60 min had no effect on reducing DON. However, in the current study, the difference between the two types of proofed doughs was not significant in case of wheat sourdough baking. Therefore, the effectiveness of higher temperature in reducing ENNs was not as remarkable as it was in reducing DON (Samar et al., 2001).

Contrary to ENN B and ENN B1, the concentration of BEA was generally not affected by the dough kneading and fermentation processes. Aside from the different chemical structures of BEA and ENNs, their different contamination patterns (BEA was artificially added and ENNs were naturally present) might also have contributed to their difference in behavior. As pointed out by Hazel and Patel (2004), the pattern of *Fusarium* infection in the cereal kernels is crucial to the fate of mycotoxins during food processing. In their report, natural contamination during growth in the field or artificial contamination of harvested grains in the laboratory were assumed to be different. Accordingly, artificial addition of mycotoxins could lead to even remarkable differences as is the case in this study.

During the final baking procedure (200 ℃ for 25 min), stronger reductions of ENN B and ENN B1 were found in crust than in crumb (5% and 9%, respectively) in case of rye sourdough baking, which was obviously due to the temperature difference between the different parts of the dough being baked. As reported by Siegel et al. (2010), the outermost layer of the dough had higher temperature than the inside of the dough, therefore, the stronger reductions of ENNs found in crust suggest that these mycotoxins were less stable under higher temperature. In a previous report, stronger decreases in

nivalenol, 3-acetyl DON, DON and ochratoxin A were found in the bread crust compared to the inner part of the bread (Valle-Algarra et al., 2009).

Overall, for both wheat and rye sourdough bread making, 25–41% of ENN B, ENN B1 and BEA were reduced from the starting materials (flour and sourdough starter) during the whole process, with the exception of BEA in crumb in case of rye sourdough baking. In comparison, Vaclavikova et al. (2013) found that ENN B and ENN B1 dropped to 60% and 50%, respectively, of their original levels in wheat flour, after a straight dough baking procedure which included a total fermentation time of 95 min at 30 ℃, and final baking of 14 min at 240 ℃. Possibly, the longer fermentation time and higher baking temperature were more effective in removing ENNs.

4.5 Occurrence of ENNs and BEA in Chinese medicinal herbs

Of the 60 medicinal herbs investigated, 25% were found to be contaminated with at least one of ENNs and BEA, and total contamination levels of these mycotoxins varied between 2.5 and 751 μg/kg, with a mean value of 126 μg/kg in the positive samples.

Until now, no data is available concerning the contaminations of ENNs and BEA in medicinal herbs to the best of my knowledge. Several previous findings have reported the occurrence of these mycotoxins in cereals. The levels of ENNs and BEA found in the 60 medicinal herbs were comparable to those reported by Sørensen et al. (2008) in whole maize harvested in 2005 and maize silage from Denmark, with levels of ENNs and BEA up to 489 and 73 μg/kg, respectively. Compared to the ENNs found in breakfast cereals and infant cereals from Morocco (Mahnine et al., 2011), where hundreds of mg/kg of ENNs were detected, the levels of ENNs in the present study were considerably lower. As for BEA, higher concentrations were also found by the latter authors (Mahnine et al., 2011), with a maximum level of 10.6 mg/kg. Although BEA was only present in 5.8% of all 68 samples (Mahnine et al., 2011), it does not necessarily mean that the frequency of BEA was lower than that in the present study, as the LOQ (0.5 mg/kg) in their report was much higher than that in this study.

Information regarding contamination of ENNs and BEA in medicinal herbs is important because half of all healthcare delivered in China is based on traditional Chinese medicines (Trucksess & Scott, 2008). Besides, medicinal herbs are also becoming popular as alternative medicines in developed world (Shaw, 1998). The examined 60 types of medicinal herbs are especially important as they are not only used as medicines, but also widely consumed as food. Therefore, their consumption is much higher than that of common herbal medicines.

By use of SIDAs, the ENNs and BEA in herbs were detected accurately and precisely without tedious cleanup. As pointed out by Han et al. (2010), the variable matrix effects have limited the application of LC-MS/MS for the analysis of traditional Chinese medicines, where matrix calibration would be unpractical as there are thousands of traditional Chinese medicines, and that the best way to solve this problem is probably by using stable isotope internal standards. Therefore, they used [$^{13}C_{17}$]-aflatoxin B1 as internal standard for the determination of 6 aflatoxins, and [$^{13}C_{18}$]-zearalenone for the determination of zearalenone and its 5 metabolites (Han et al., 2010 & 2011). However, as aflatoxin B1 does not behave exactly the same way as the other 5 aflatoxins, and zearalenone not the same as its

5 metabolites, their determinations might not be as accurate as they would be if each and every one of the aflatoxins and zearalenone metabolites had corresponding internal standards. This problem was solved in the current study by using $^{15}N_3$-labeled ENNs A, A1, B, B1, and BEA as internal standards for the respective ENNs and BEA.

5. Conclusion

The emerging mycotoxins ENNs and BEA are produced by various fungi belonging to the *Fusarium* genus. In the recent decades, there have been increasing reports regarding the contamination of these mycotoxins in cereals and cereal-based food, with especially high incidences and contamination levels being found in samples from Norway (Uhlig et al., 2006), Denmark (Sørensen et al., 2008), and Spain (Meca et al., 2010c).

In the present work, the analysis of cereals and related food from Germany and medicinal herbs from China has added further evidence to the worldwide contamination of ENNs and BEA.

Among a total of 65 cereals and related food samples from Germany, ENN B (87.7%) and ENN B1 (83.1%) were detected frequently, followed by ENN A1 (52.3%) and ENN A (16.9%). High concentrations of ENN B and ENN B1 were found in barley malt and wheat grain samples, with mean levels of 658–3668 µg/kg. In rye bread, wheat flour (conventional), and spaghetti (conventional) samples, levels of ENN B and ENN B1 ranged from 54–263 µg/kg.

In addition, 25% of the 60 different Chinese medicinal herbs examined in this study were contaminated by at least one of ENNs and BEA, with total contamination levels varying between 2.5 and 751 µg/kg, and a mean value of 126 µg/kg in the positive samples. In China, the consumption of these herbs is high because they are used both as medicines and food. For example, ginger is commonly used in its fresh form or as spice in cooking, and in the present study, the dried ginger sample was one of the most highly contaminated samples (containing up to 355 µg/kg of ENN A). Other contaminated herbs found in this study such as wrinkled giant hyssop herb (99 µg/kg of BEA) and heartleaf houttuynia herb (89 µg/kg of BEA) are consumed as vegetables in certain areas of China. These data suggest a common exposure of humans to ENNs and BEA, of which a number of biological activities have been reported, indicating possible toxicity of these mycotoxins. However, the existing toxicological data mostly deal with their *in vitro* activities, whereas valid *in vivo* data is lacking. Considering that ENNs and BEA have similar chemical structures to that of tentoxin, which is a cyclic peptide, and that similarly to tentoxin, ENNs and BEA also show no genotoxic properties, the 1500 ng/kg bw per day exposure threshold of toxicological concern (TTC) value of tentoxin (EFSA, 2011) can be adopted for ENNs and BEA to assess their health risks. Based on the contamination levels of ENNs and BEA in cereals and cereal products from Germany (including all the grains of

wheat, oat, rice, and maize, flour of maize and wheat, maize grits, oat flakes, and spaghetti samples listed in **Table 3-14**), and the average daily consumption of cereals and cereal products (70 and 58 g per day for man and woman, respectively) according to German Nutrition Society (DGE, 2012), the mean chronic dietary exposures of the single ENNs A1, B, and B1 for consumers in Germany are estimated to be more than 6-fold lower than the TTC value, whereas those of ENN A and BEA are negligibly low as their contamination levels were below limits of detection. Similarly, based on the mean contamination levels of ENNs and BEA in wheat and rye bread from **Table 3-14**, and given that in average, 158 and 111 g per day of bread are consumed by man and woman, respectively, in Germany (DGE, 2012), the mean chronic dietary exposures of single ENNs A1, B and B1 are estimated to be more than 4-fold lower than the TTC value, while those of ENN A and BEA are again negligibly low as their contamination levels were below limits of detection. In addition, if all single ENNs are summarized, a sum ENNs exposure of 785 and 719 ng/kg bw for man and woman, respectively, can be calculated and is about one half of the TTC. Therefore, ENNs and BEA are considered unlikely to be of concerns to human health. The mean dietary exposures of ENNs and BEA for German consumers are summarized in **Table 5-1**. To better assess the risks of ENNs and BEA to humans and animals, research on their *in vivo* toxicity, especially chronic effects, are needed urgently.

The study of sourdough bread making revealed that overall, the concentrations of ENNs and BEA in bread were 25−41% lower than those in the starting materials. Therefore, the toxic risks of sourdough bread would be generally lower compared to the original wheat and rye flours. However, as little is known about the degradation products of ENNs and BEA, more research will be necessary in order to assess their risks to human health.

Regarding the risks of ENNs and BEA from beer consumption, the study on their fate during beer making showed that the carryover of these mycotoxins was less than 0.2% from barley to beer. Even starting from barley grains containing 119 mg/kg of ENN B, only 74 µg/kg was detected in the final beer. Therefore, the contamination of ENNs and BEA on barley grains should pose little if any risk to beer drinkers. However, the highly contaminated spent grains (up to 251.5 mg/kg) could pose possible risks if fed to animals.

Table 5-1. Mean dietary exposures of ENNs and BEA for German consumers

	Mean contamination level (ng/g food)		Mean dietary exposure (ng/kg bw per day)	
	Cereals & cereal products[a]	Bread[b]	Cereals & cereal products[a] (man/woman)[c]	Bread[b] (man/woman)[c]
ENN A	-	-	-	-
ENN A1	17	6	16/17	12/10
ENN B	213	155	205/206	336/287
ENN B1	102	55	98/98	118/101
BEA	-	-	-	-

[a] including all the grains of wheat, oat, rice, and maize, flour of maize and wheat, maize grits, oat flakes, and spaghetti samples listed in **Table 3-14**.

[b] including both wheat bread and rye bread samples listed in **Table 3-14**.

[c] calculated based on body weights of 73 and 60 kg for man and woman, respectively (Valentin, 2002).

-, below limit of detection.

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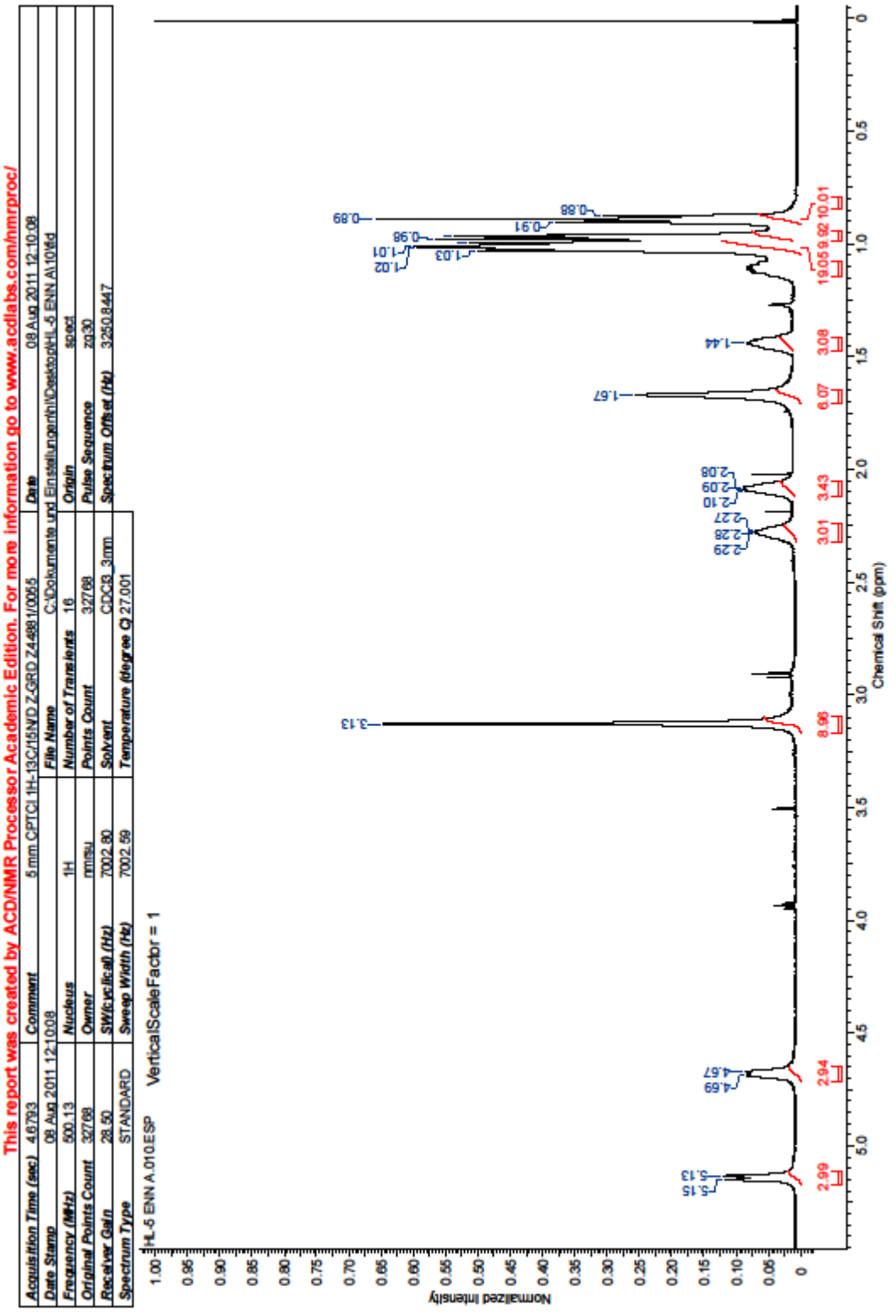
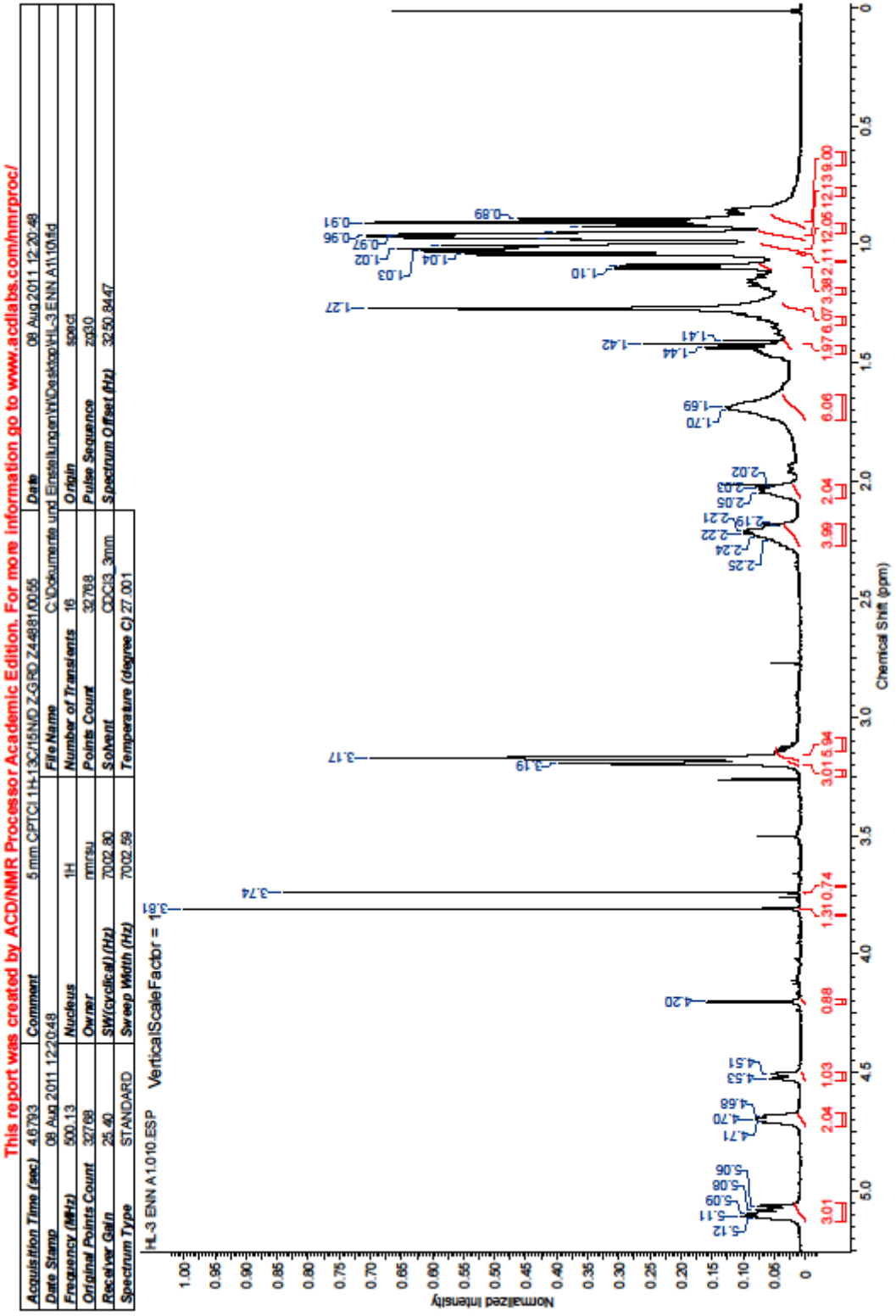
7. Appendix

^{1}H-NMR spectrum of [^{15}N]$_3$-ENN A



^{1}H-NMR spectrum of [^{15}N]$_3$-ENN A1



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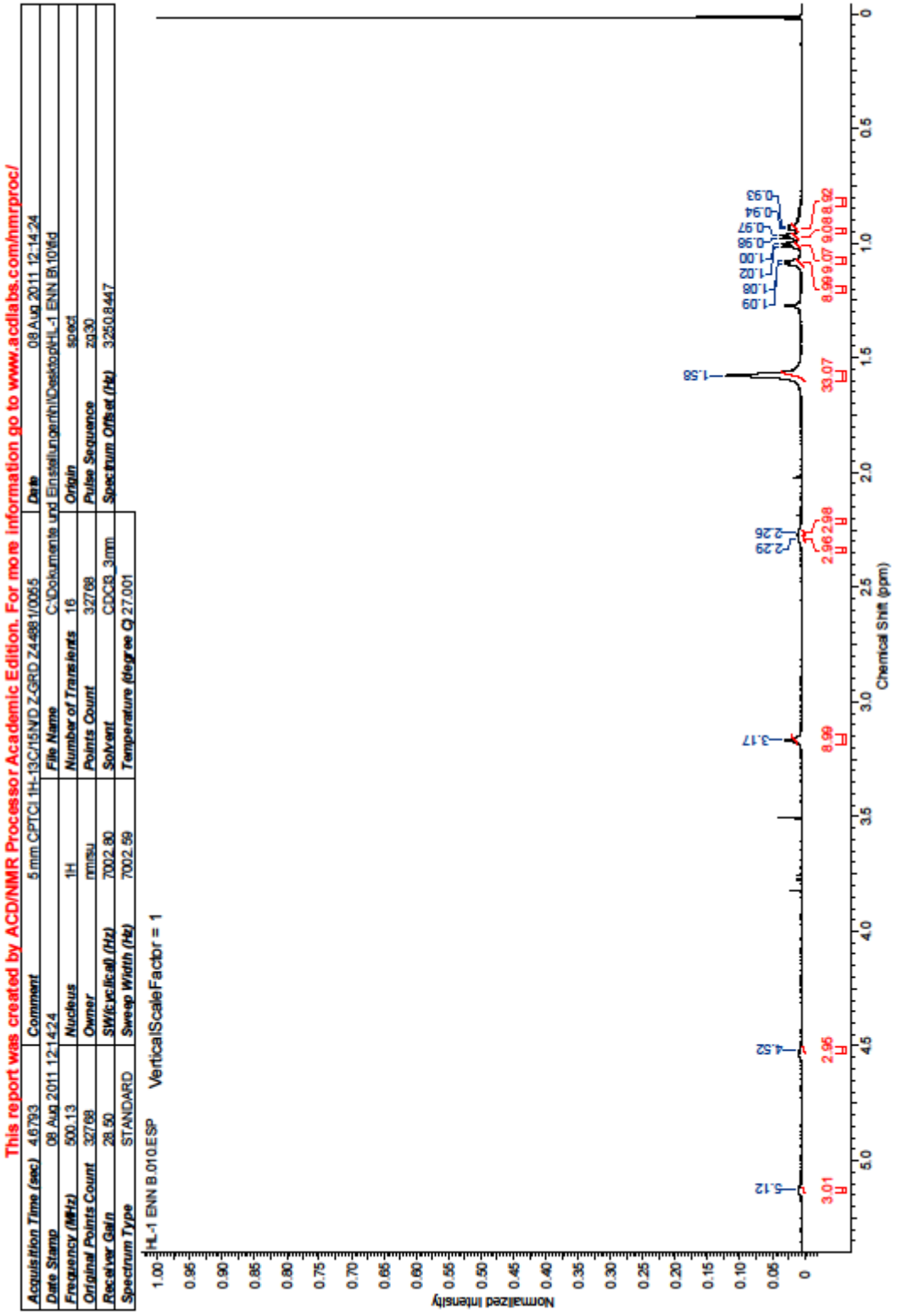

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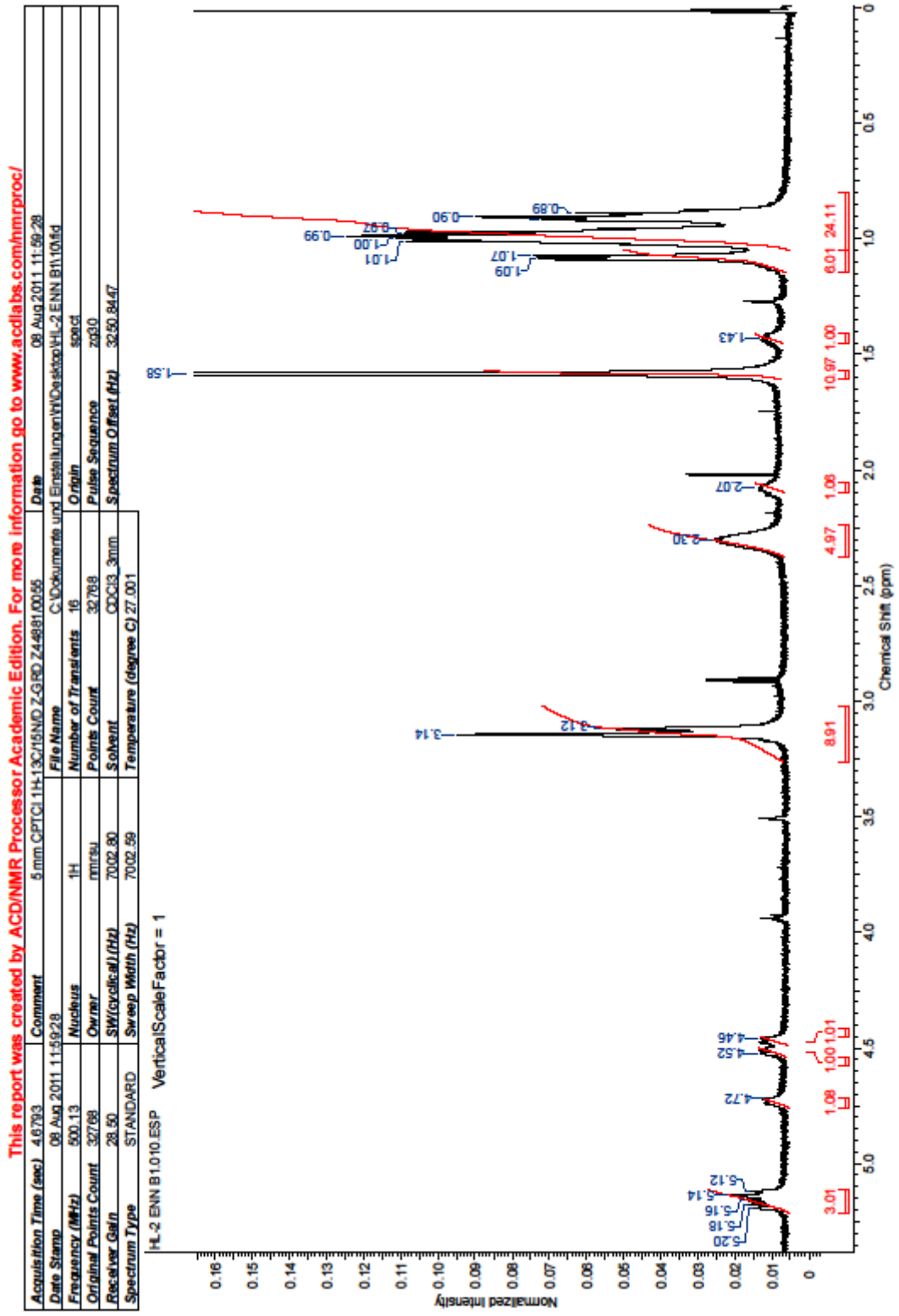

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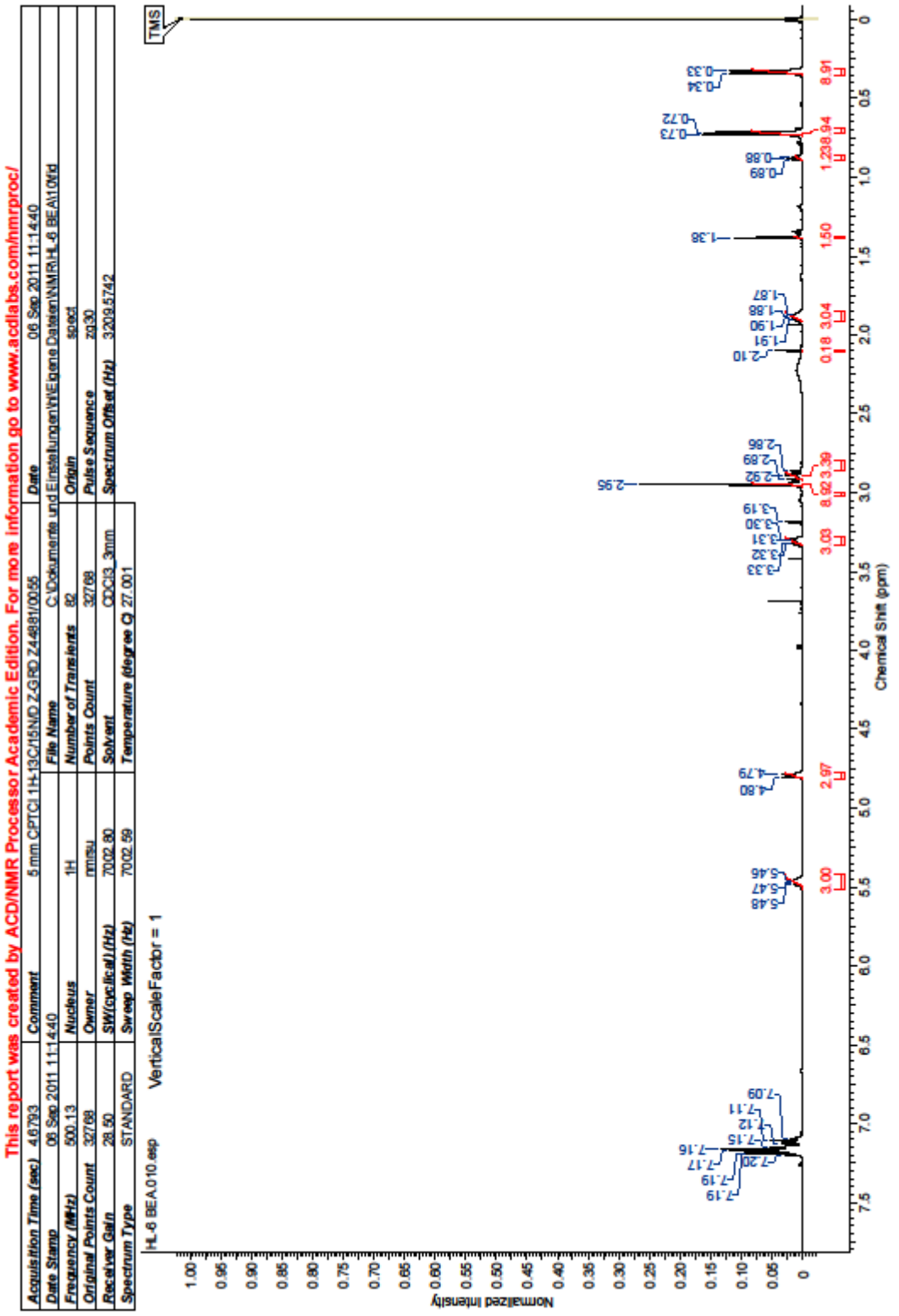
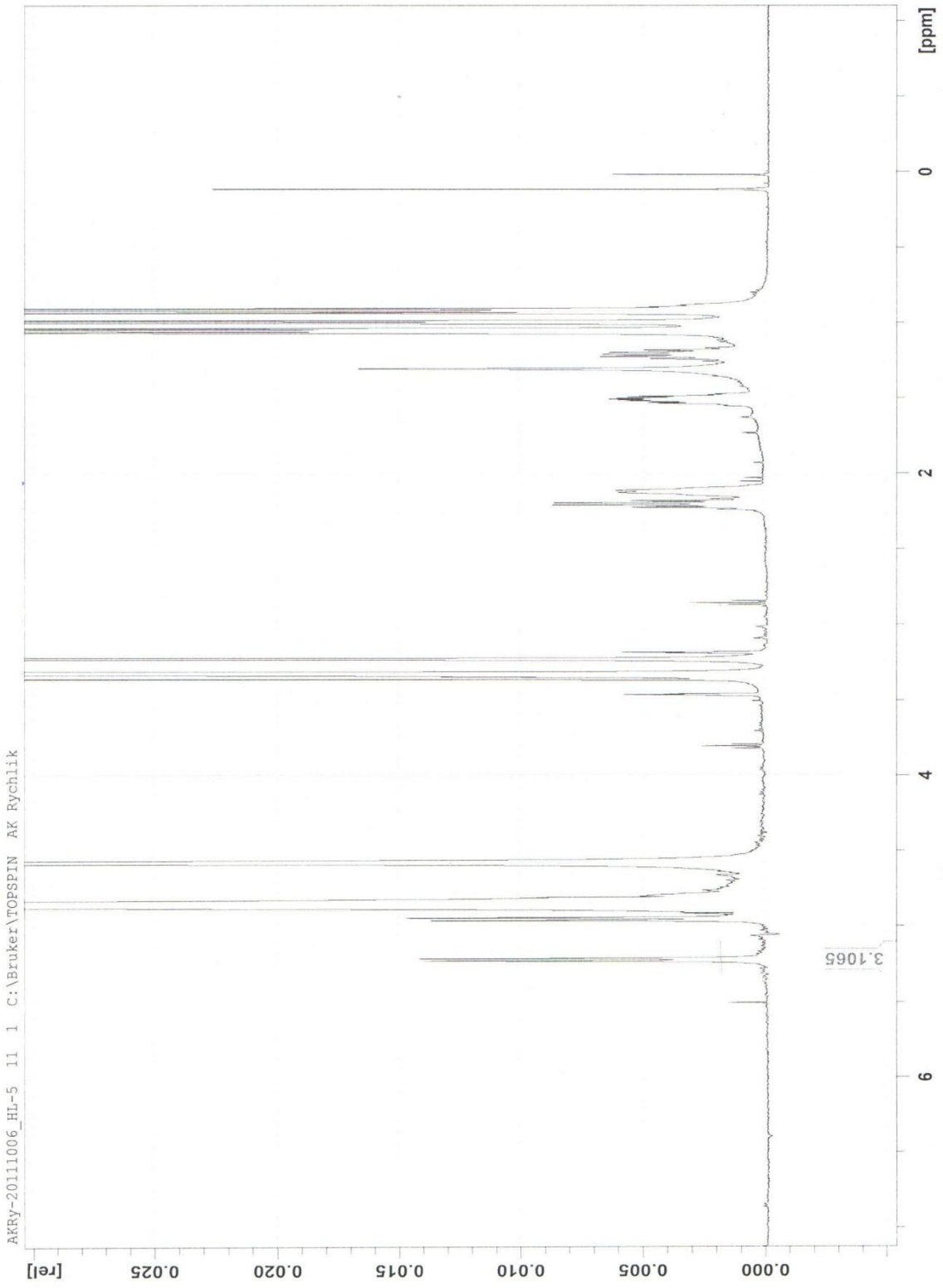


qNMR spectrum of $[^{15}N]_3$-ENN A



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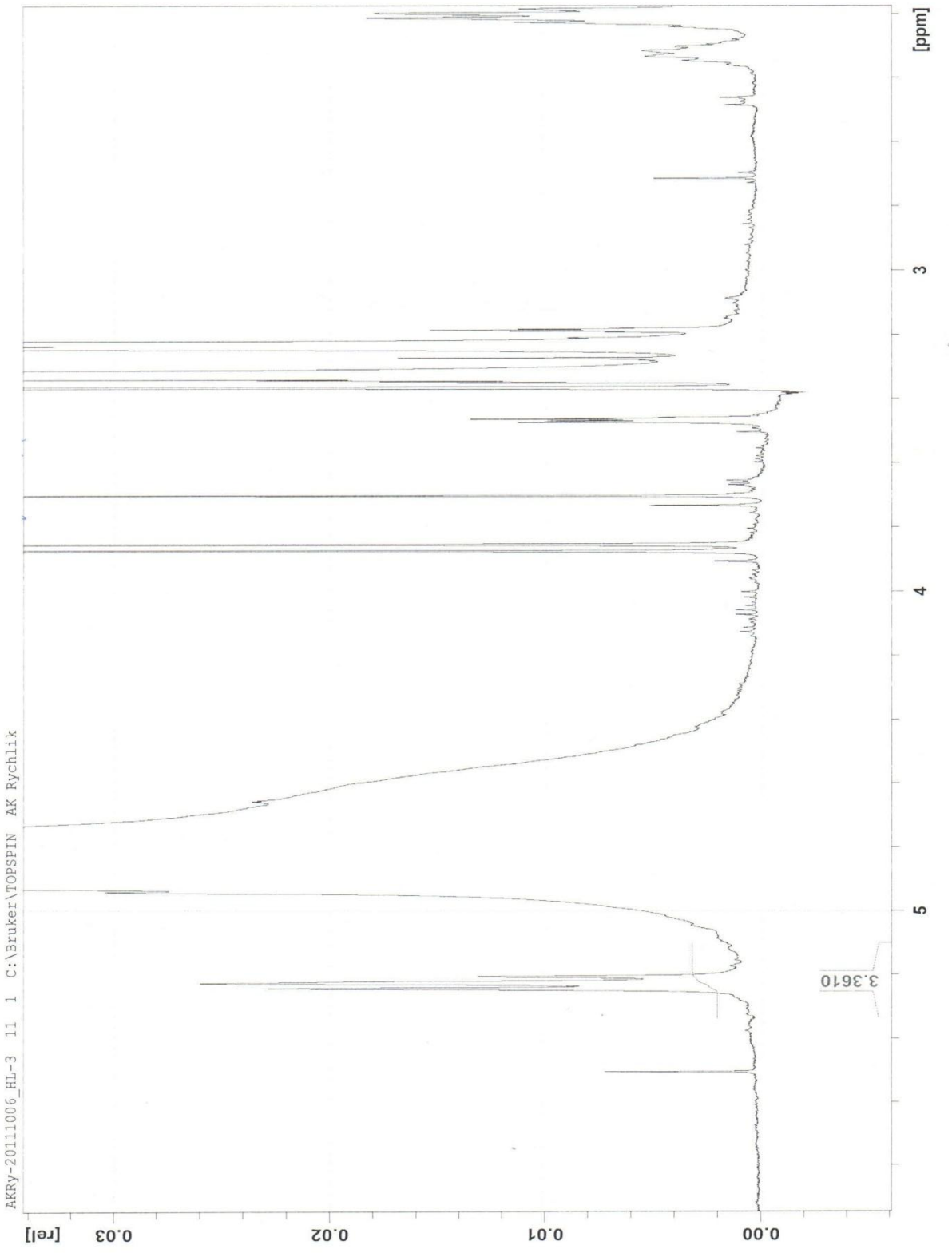

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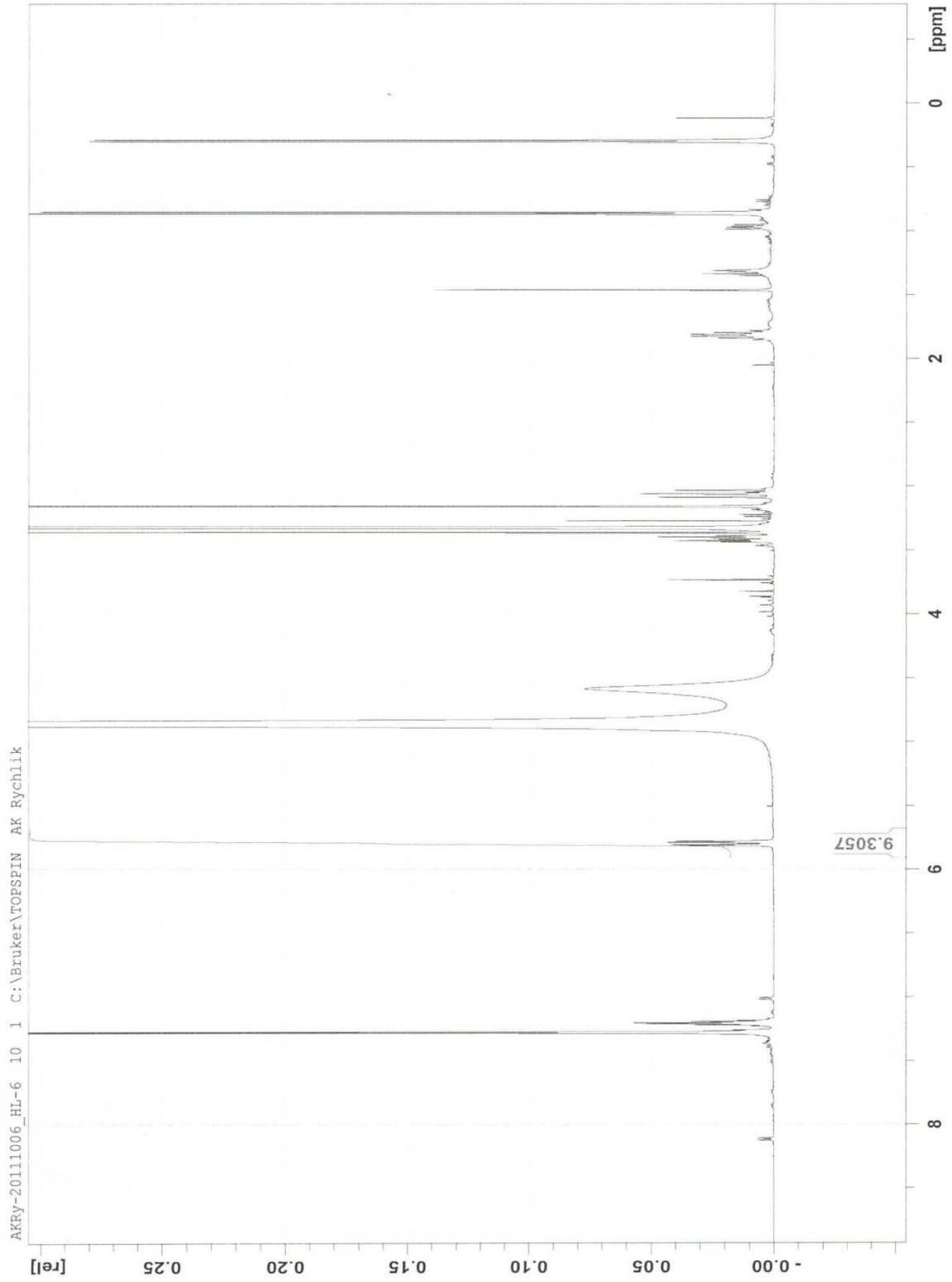

8. List of tables

9. List of figures

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11. Curriculum vitae

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Publikationen

1. **Hu, Ling**; Rychlik, M. (2012). Biosynthesis of $^{15}N_3$-labeled enniatins and beauvericin and their application to stable isotope dilution assays. *Journal of Agricultural and Food Chemistry*, 60, 7129−7136.
2. **Hu, Ling**; Gastl, M.; Linkmeyer, A.; Hess, M.; Rychlik, M. (2014). Fate of enniatins and beauvericin during the malting and brewing process determined by stable isotope dilution assays. *LWT - Food Science and Technology*, 56, 469−477.
3. **Hu, Ling**; Koehler, P.; Rychlik, M. (2013). Effect of sourdough processing and baking on the content of enniatins and beauvericin in wheat and rye bread. *European Food Research and Technology*, published online, DOI 10.1007/s00217-013-2133-4.
4. Linkmeyer, A.; Götz, M.; **Hu, Ling**; Asam, S.; Rychlik, M.; Hausladen, H.; Hess, M.; Hückelhoven, R. (2013). Assessment and introduction of quantitative resistance to Fusarium head blight in elite spring barley. *Phytopathology*, 103, 1252−1259.

Konferenzbeiträge

1. 05−09/11/2012, WMF meets IUPAC 2012-The World Mycotoxin Forum, Rotterdam, the Netherlands. Presentation of poster
2. 27−31/05/2013, ISM-MycoRed International Conference Europe 2013, Global Mycotoxin Reduction Strategies, Martina Franca, Italy. Presentation of poster